

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: T. Takagi et al. Attorney Docket No.: DAISAN126512
Application No.: 10/555076 Art Unit: 1617 / Confirmation No.: 3081
Filed: March 2, 2006 Examiner: T.E. Betton
Title: ADIPONECTIN PRODUCTION ENHANCER

RESPONSE FILED WITH RCE

Seattle, Washington 98101

March 6, 2009

TO THE COMMISSIONER FOR PATENTS:

Claims 41, 43-48, 55-57, and 59-62 are pending in the application and have been rejected. Reconsideration and allowance of Claims 41, 43-48, 55-57, and 59-62 in view of the following remarks is respectfully requested.

The Claimed Invention

Claims 41, 43-48, 55-57, and 59-62 are pending. Claims 41 and 48 are the pending independent claims.

Claim 41 is directed to a method for increasing adiponectin production that includes administering one or more HMG-CoA reductase inhibitor(s). Claims 43-47, 57, 59, and 60 depend from Claim 41.

Claim 48 is directed to a method for treating hypoadiponectinemia that includes administering one or more HMG-CoA reductase inhibitor(s). Claims 55-57, 61, and 62 depend from Claim 48.

The Rejection of Claims 41-62 Under 35 U.S.C. § 112, First Paragraph

Claims 41-62 have been rejected under 35 U.S.C. § 112, first paragraph, for failing to enable the skilled person to use the invention commensurate in scope with the claims. Withdrawal of the rejection is requested for the following reasons.

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1420 Fifth Avenue
Suite 2800
Seattle, Washington 98101
206.682.8100

Claims 41 and 48 are the remaining pending independent claims. Claim 41 is directed to a method for increasing adiponectin production that includes administering one or more HMG-CoA reductase inhibitor(s). Claims 43-47, 57, 59, and 60 depend from Claim 41. Claim 48 is directed to a method for treating hypoadiponectinemia that includes administering one or more HMG-CoA reductase inhibitor(s). Claims 55-57, 61, and 62 depend from Claim 48.

Regarding Claim 41 and its dependent claims, the Examiner's concern regarding identifying a subject population for treatment is not relevant to the recited method for increasing adiponectin production. The specification describes the relationship between adiponectin and diabetes, and that increasing adiponectin production is useful for the treatment or prevention of diabetes and insulin resistance syndrome, and complications caused thereby. See page 3, lines 17, to page 4, line 23. Therefore, the skilled person in the art of diabetes diagnosis can determine those having depressed adiponectin levels and then address that deficiency by the claimed method.

Similarly, with regard to Claim 48, the application as filed describes disease states associated with decreased blood adiponectin concentrations, including hypoadiponectinemia. See, for example, page 2, lines 9-30. Therefore, applicants submit that it is well within the purview of the skilled person to diagnose a person suffering from depressed adiponectin level (hypoadiponectinemia) and then address that deficiency by the claimed method.

Hypoadiponectinemia is a well-known condition that is readily identified and diagnosed by a person skilled in the art (i.e., a medical doctor in the relevant field). The application has provided sufficient support for the inhibitory effect of HMG-CoA reductase inhibitors on the diseases recited in Claims 41 and 48, as evidenced by Example 1, "Adiponectin production enhancing action (in vitro)," and Example 2, "Adiponectin production enhancing action (in vivo) and glucose uptake enhancing action." Furthermore, M.P.E.P. 2164.02 titled "Working Example" allows the correlation between *in vitro* and *in vivo* animal model assays and a claimed

method of use "if the art is such that a particular model is recognized as correlating to a specific condition."

In applicants' response filed November 20, 2007, the Examiner was provided evidence of the relationship between adiponectin and certain diseases: (1) Shimomura et al., "Pathophysiological Significance of Adiponectin," *Medical Molecular Morphology* 40:55-67, 2007, attached in the previous response as Exhibit A; and (2) Han et al., "Adiponectin and Cardiovascular Disease," *Journal of the American College of Cardiology* 49:531-538, 2007, attached in the previous response as Exhibit B.

The Shimomura publication shows the clinical significance of adiponectin and the relationship between adiponectin and several diseases (obesity, cardiovascular disease, hypertension and dyslipidemia, metabolic syndrome, inflammation, cancer, and other diseases). See pages 58 and 59. The Han publication describes the relationship between adiponectin and cardiovascular disease.

As evidenced by these publications, the skilled person would understand that the experimental results set forth in the application as originally filed establish the correlation between the *in vitro* and *in vivo* animal model assays in the application and the claimed methods. The mouse model described in Example 2 of the application is recognized in the art as correlating with specific conditions of diabetes, such as insulin tolerance and glucose uptake, as well as demonstrating that adiponectin levels can be measured in a warm-blooded animal. Moreover, the specification cites references that show that mouse models for studying adiponectin and glucose metabolism were well known in the art (see page 3, lines 23-30). Further, the mouse model is well established as a model for testing the effect of statins on lipid homeostasis. Moreover, as described in the specification, the measurement of adiponectin levels in human plasma and mouse blood are routine and well known in the art (see, for example, page 2, lines 9-20; Example 2, page 28, lines 5-9).

Because the level of predictability in the art of selecting subjects with low adiponectin levels is high, and the quantity of experimentation required to identify subjects with reduced adiponectin levels is not undue, the application as originally filed provides a disclosure that enables the skilled person to make and use the claimed invention without undue experimentation. See *in re Wands*, 858 F.2d 731 (holding that the specification was enabling because "there was considerable direction and guidance" in the specification; there was "a high level of skill in the art at the time the application was filed;" and "all of the methods needed to practice the invention were well known.") Further, no evidence has been submitted supporting the Examiner's conclusion concerning the alleged lack of enablement. Accordingly, the application satisfies the enablement requirement. Therefore, withdrawal of the rejection is respectfully requested.

The Rejection of Claims 41-62 Under 35 U.S.C. § 103(a)

Claims 41-62 have been rejected under 35 U.S.C. § 103(a) as being unpatentable as obvious over the combined teaching of U.S. Patent No. 6,130,214, issued to Lohray et al., in view of U.S. Patent No. 6,384,062, issued to Ikeda et al. The Examiner further relies on Schulze et al., "Adiponectin and Future Coronary Heart Disease Events Among Men With Type 2 Diabetes," *Diabetes* 54:534-539, 2005, printed pages 1-6.

As noted above, Claims 42, 49-54, and 58 have been canceled. Claims 41 and 48 are the remaining pending independent claims. Claim 41 is directed to a method for increasing adiponectin production that includes administering one or more HMG-CoA reductase inhibitor(s). Claims 43-47, 57, 59, and 60 depend from Claim 41. Claim 48 is directed to a method for treating hypoadiponectinemia that includes administering one or more HMG-CoA reductase inhibitor(s). Claims 55-57, 61, and 62 depend from Claim 48.

Relying on the publication to Schulze et al. (Adiponectin and Future Coronary Heart Disease Events among Men With Type 2 Diabetes, *Diabetes* 54:534-539, 2005), the Examiner is of the view that adiponectin was well known in the art as a major modulator of insulin resistance and dyslipidemia. The Examiner is also of the view that HMG-CoA reductase inhibitors are

effective against dyslipidemia and conditions thereof. The Examiner then concludes that by virtue of the mechanism of action of agents such as pravastatin, adiponectin is likely to increase in instances as a typical characteristic and property of HMG-CoA reductase inhibitor treatment.

To assist the Examiner in further understanding the state of the art (the knowledge of the skilled person) and the relationship between adiponectin and HMG-CoA reductase inhibitors, applicants provide five publications: (1) Koh, K.K. et al., "Additive Beneficial Effects of Losartan Combined With Simvastatin in the Treatment of Hypercholesterolemic, Hypertensive Patients," *Circulation* 110:3687-92, 2004, attached as **Exhibit A**; (2) Forst, T., et al., "Effect of Simvastatin and/or Pioglitazone on Insulin Resistance, Insulin Secretion, Adiponectin, and Proinsulin Levels in Nondiabetic Patients at Cardiovascular Risk--the PIOSTAT Study," *Metabolism* 56:491-496, 2007, attached as **Exhibit B**; (3) Shetty, G.K. et al., "Circulating Adiponectin and Resistin Levels in Relation to Metabolic Factors, Inflammatory Markers, and Vascular Reactivity in Diabetic Patients and Subjects at Risk for Diabetes," *Diabetes Care* 10:2450-7, 2004, attached as **Exhibit C**; (4) Koh, K.K. et al., "Additive Beneficial Effects of Fenofibrate Combined with Atorvastatin in the Treatment of Combined Hyperlipidemia," *Journal of the American College of Cardiology* 45:1649-53, 2005, attached as **Exhibit D**; and (5) Sakamoto, K., et al., "The Effect of 6 Months of Treatment with Pravastatin on Serum Adiponectin Concentrations in Japanese Patients with Coronary Artery Disease and Hypercholesterolemia: A Pilot Study," *Clinical Therapeutics* 28(7):1012-1021, July 7, 2006, attached as **Exhibit E**.

The above references provide evidence that, as of the priority date of the present application, one of skill in the art would not expect statins to increase adiponectin levels in a warm-blooded animal. For example, The Koh (2004) publication shows that treatment of hypercholesterolemic, hypertensive patients with simvastatin did not significantly increase plasma levels of adiponectin or insulin sensitivity, whereas treatment with the angiotensin II type 1 receptor blocker losartan significantly increased both adiponectin levels and insulin

sensitivity, and the beneficial effects of losartan therapy on adiponectin levels and insulin sensitivity did not increase further in combination with simvastatin (pages 3690-91, Figures 4 and 5). The Forst publication shows that treatment of patients with increased cardiovascular risk with simvastatin resulted in a significant reduction in plasma adiponectin levels (pages 493-494, Figure 1). The Shetty publication shows that treatment of patients with diabetes or at high risk for type 2 diabetes with atorvastatin did not significantly change serum adiponectin levels, indicating that atorvastatin had no specific effect on adiponectin levels (page 2453 and Table 5). The Koh (2005) publication shows that treatment of patients with combined hyperlipidemia with atorvastatin did not significantly increase plasma adiponectin levels or insulin resistance (page 1652, Table 3, Figure 4). The Sakamoto publication shows that treatment of patients with coronary artery disease with pravastatin increased serum adiponectin levels after 6 months of treatment. The Sakamoto publication was published after the filing date of the present U.S. application.

As evidenced by these publications, all of which have a publication date later than the filing date of the international application that forms the basis of the pending U.S. application, the skilled person would not have a reasonable expectation that treatment of patients having dyslipidemia or diabetes with HMG-CoA reductase inhibitors would result in increased production of adiponectin. Therefore, the Examiner's conclusion that the mechanism of action of statins would necessarily lead to increases in adiponectin levels is not supported by the knowledge of one skilled in the art as of the filing date of the present application. Indeed, the weight of the evidence supports the novelty and non-obviousness of the claimed methods, because four out of five studies that measured adiponectin levels after treatment with various HMG-CoA reductase inhibitors found either no increase or a decrease in adiponectin levels.

In the Final Office Action dated February 8, 2008, the Examiner stated that Ikeda provides the motivation to combine the compositions and methods of the Lohray reference with the compositions and methods of the Ikeda reference because the Ikeda reference teaches the

administration of pravastatin for various disease states and conditions, which makes the instant claimed methods obvious. As described in detail in the previous amendment, filed July 8, 2008, and entered on September 10, 2008, neither the Lohray nor Ikeda reference describes, teaches, or provides any motivation to arrive at a method for either increasing adiponectin production or treating hypoadiponectinemia. In addition to the remarks filed on July 8, 2008, which are hereby incorporated by reference, applicants submit that the Ikeda reference does not provide the alleged motivation to arrive at the claimed methods, for at least the following reasons.

The experimental examples of the Ikeda reference disclose treatment of genetically obese and diabetic Wistar fatty rats with pioglitazone hydrochloride (an insulin sensitivity enhancer) in combination with an alpha-glucosidase inhibitor (voglibose) (Example 1), or pioglitazone hydrochloride in combination with an insulin secretion enhancer (glibenclamide) (Example 2), and the effects of the treatments on plasma glucose levels. However, as noted above, the Ikeda reference does not disclose that a insulin sensitivity enhancer, such as pioglitazone, in combination with a statin has any effect on adiponectin levels, or that pioglitazone in combination with a statin has any effect whatsoever on insulin sensitivity or any other parameter related to diabetes. Further, the Forst reference (submitted as Exhibit B) discloses that pioglitazone produced significant increases in plasma adiponectin levels, and significant improvements in insulin sensitivity as well as blood glucose and insulin profiles during the oral glucose tolerance test. In contrast, simvastatin produced a decrease in plasma adiponectin and no improvement in insulin resistance or glucose and insulin profiles during the oral glucose tolerance test. Pioglitazone in combination with simvastatin did not improve the results of using pioglitazone alone.

The Koh (2005) reference (submitted as Exhibit D) discloses that treatment with fenofibrate, a PPAR-alpha agonist, resulted in both an increase in adiponectin levels and insulin sensitivity, whereas treatment with atorvastatin alone did not increase either adiponectin levels or insulin sensitivity. The combination of fenofibrate and atorvastatin did not result in significant

increases in adiponectin or insulin sensitivity over fenofibrate alone. Thus, both the Forst and Koh references teach that combinations of an insulin sensitivity enhancer with a statin are not effective in increasing adiponectin levels, which directly rebuts the teachings of the Ikeda reference, which states that "[t]he pharmaceutical composition of the present invention shows a marked synergistic effect compared with administration of either active component alone," and allows each component to be used at reduced dosages to reduce unwanted side effects (column 15, lines 23-41). In this case, the present methods provide the benefit of increasing adiponectin production without exposing the patient to additional compounds, such as those of the Lohray and Ikeda references, thereby eliminating the potential side effects of a second drug. Therefore, the present methods provide for the omission of additional drugs that are unnecessary for increasing adiponectin production. See MPEP 2144.04(II)(B) (the omission of an element with retention of the elements function is an indicia of nonobviousness).

Further, the Forst and Koh (2004, 2005) references teach away from the assertion by the Examiner that the Ikeda reference provides motivation for arriving at the instant claimed methods, and provide evidence that one of skill in the art would not have a reasonable expectation of success in increasing adiponectin levels using a statin alone. Moreover, the Koh (2005) reference teaches that "[t]he effects of statins on insulin resistance are controversial" (page 1649, right hand column), providing evidence that the prior art was unpredictable regarding the role of statins in treatments for diabetes.

In summary, it is submitted that a *prima facie* case for obviousness of the claimed methods has not been established, because the cited references do not teach or remotely suggest increasing adiponectin production by administering an HMG-CoA reductase inhibitor, the evidence provided above as Exhibits A-D indicates that the role of statins in diabetes were unpredictable as of the priority date of the present application, and further indicates that one of skill in the art would not have a reasonable expectation of success because the above references teach away from the claimed methods.

CONCLUSION

In view of the above amendments and foregoing remarks, Claims 41, 43-48, 55-57 and 59-62 are believed to be in condition for allowance. If any issues remain that may be expeditiously addressed in a telephone interview, the Examiner is encouraged to telephone applicants' attorney at 206.695.1755.

Respectfully submitted,

CHRISTENSEN O'CONNOR
JOHNSON KINDNESS^{PLLC}



George E. Renzoni, Ph.D.
Registration No. 37,919
Direct Dial No. 206.695.1755

GER/sjt

EXHIBIT A

Additive Beneficial Effects of Losartan Combined With Simvastatin in the Treatment of Hypercholesterolemic, Hypertensive Patients

Kwang Kon Koh, MD; Michael J. Quon, MD, PhD; Seung Hwan Han, MD; Wook-Jin Chung, MD; Jeong Yeal Ahn, MD; Yiel-Hea Seo, MD; Moon Ho Kang, MD; Tae Hoon Ahn, MD; In Suck Choi, MD; Eak Kyun Shin, MD

Background—Biological mechanisms underlying statin and angiotensin II type 1 receptor blocker therapies differ. Therefore, we compared vascular and metabolic responses to these therapies either alone or in combination in hypercholesterolemic, hypertensive patients.

Methods and Results—This was a randomized, double-blind, placebo-controlled crossover trial with 3 treatment arms (each 2 months) and 2 washout periods (each 2 months). Forty-seven hypertensive, hypercholesterolemic patients were given simvastatin 20 mg and placebo, simvastatin 20 mg and losartan 100 mg, or losartan 100 mg and placebo daily during each 2-month treatment period. Losartan alone or combined therapy significantly reduced blood pressure compared with simvastatin alone. Compared with losartan alone, simvastatin alone or combined therapy significantly changed lipoproteins. All 3 treatment arms significantly improved flow-mediated dilator response to hyperemia and decreased plasma malondialdehyde and monocyte chemoattractant protein-1 levels relative to baseline measurements. However, these parameters were changed to a greater extent with combined therapy compared with simvastatin or losartan alone (both $P < 0.001$ and $P = 0.030$ for monocyte chemoattractant protein-1 by ANOVA). Combined therapy or losartan alone significantly increased plasma adiponectin levels and insulin sensitivity (determined by QUICKI) relative to baseline measurements. These changes were significantly greater than in the group treated with simvastatin alone ($P < 0.001$ for adiponectin, $P = 0.029$ for QUICKI by ANOVA).

Conclusions—Simvastatin combined with losartan improves endothelial function and reduces inflammatory markers to a greater extent than monotherapy with either drug in hypercholesterolemic, hypertensive patients. (*Circulation*. 2004; 110:3687-3692.)

Key Words: angiotensin ■ endothelium ■ hypercholesterolemia ■ hypertension ■ insulin

Hypercholesterolemia and hypertension are major public health problems that are frequently treated with statins and angiotensin II type 1 (AT1) receptor blockers, respectively. Although the mechanisms of action for these 2 classes of drugs differ, both classes have beneficial effects on the vasculature. Indeed, large-scale clinical studies have demonstrated that simvastatin, an HMG-CoA reductase inhibitor, and losartan, an AT1 receptor blocker, prevent and retard the progression of coronary heart disease.^{1,2} Hypertension and coronary heart disease are frequently associated with insulin resistance and disorders of metabolic homeostasis such as obesity and type II diabetes. The endothelial dysfunction associated with cardiovascular diseases may contribute to insulin resistance and the pathophysiology of diabetes and its vascular complications.³ In fact, large-scale clinical studies have demonstrated that simvastatin and losartan reduce the

onset of new diabetes.^{1,4} The mechanisms of this benefit may relate to the ability of these therapies to reduce insulin resistance.⁵ Moreover, it is possible that simvastatin combined with losartan therapy may have additional vascular benefits that are greater than those observed for either drug alone.

Statin reduce LDL cholesterol. In addition, they improve endothelial function via stimulation of nitric oxide (NO) synthase activity and mediate antioxidant effects that result in enhanced NO bioactivity.^{6,7} AT1 receptor blockers also improve endothelial function.^{8,9} This may be due in part to diminished intracellular production of superoxide anions via reduced activity of angiotensin II-dependent oxidases.¹⁰ Inhibition of the production of superoxide anions may limit oxidation of LDL and contribute to increased NO bioactivity by limiting oxidative degradation of NO.⁷ Thus, AT1 receptor

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From Cardiology (K.K.K., S.H.H., W.-J.C., T.H.A., I.S.C., E.K.S.), Laboratory Medicine (J.Y.A., Y.-H.S.), and Endocrinology (M.H.K.), Gachon Medical School, Incheon, Korea, and Diabetes Unit, Laboratory of Clinical Investigation, NCCAM, NIH (M.J.Q.), Bethesda, Md.

Correspondence to Kwang Kon Koh, MD, PhD, FACC, FAHA, Professor of Medicine, Director, Vascular Medicine and Atherosclerosis Unit, Cardiology, Gil Heart Center, Gachon Medical School, 1198 Kuwol-dong, Namdong-gu, Incheon, Korea. E-mail kwangk@gil.com
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TABLE 1. Baseline Characteristics of the Study Population

Age, y	57±2
Sex, M:F	20:27
BMI, kg/m ²	25.2±0.5
Risk factors, n (%)	
Current smoking	11 (23)
Medications, n (%)	
β-Adrenergic blockers	10 (21)
Calcium channel blockers	6 (13)

BMI indicates body mass index. Values are expressed as mean±SEM when appropriate. n=47.

blockers may inhibit both LDL oxidation and atherosclerosis.¹¹

The endothelial dysfunction associated with diabetes, obesity, metabolic syndrome, and other insulin-resistant states is characterized by impaired NO release from endothelium.¹² Thus, improvement in endothelial function is predicted to enhance insulin sensitivity, and this may be a mechanism by which simvastatin and losartan decrease the incidence of new-onset diabetes. Adiponectin is one of a number of proteins secreted by adipose cells that may couple regulation of insulin sensitivity with energy metabolism and serve to link obesity with insulin resistance.¹³ In humans, plasma levels of adiponectin are negatively correlated with adiposity, and decreased plasma adiponectin levels are observed in patients with diabetes and those with coronary artery disease.^{14,15} Thus, decreased levels of adiponectin may play a key role in the development of insulin resistance. In addition, adiponectin also possesses antiatherogenic properties.^{16,17}

Because the impact of simvastatin and losartan therapies on NO bioactivity and its subsequent effects on oxidant stress, inflammation, endothelial function, and insulin resistance may differ, we hypothesized that combined therapy may have additive beneficial effects that are greater than those observed with either simvastatin or losartan therapy alone in hypercholesterolemic, hypertensive patients.

Methods

Study Population and Design

Fifty hypercholesterolemic, hypertensive patients (LDL cholesterol levels ≥100 mg/dL) participated in this study. We defined hypertension as systolic and diastolic blood pressure ≥140 or ≥90 mm Hg, respectively. We excluded patients with severe hypertension, unstable angina, or acute myocardial infarction. To minimize acute side effects to losartan, study medication was titrated from 50 to 100 mg upward over a 2-week period if no hypotension (systolic blood pressure <100 mm Hg) was noted. At the end of this time, participants were receiving either placebo or losartan 100 mg/d. Of 50 patients, 47 tolerated losartan 100 mg with regard to maintaining systolic blood pressure >100 mm Hg for 3 hours after drug administration and experienced no adverse effects from therapy. One patient was hypotensive, and the other 2 patients suffered from dry cough. Thus, a total of 47 patients' data were analyzed. The clinical characteristics of these patients are summarized in Table 1. Patients were randomly assigned to one of the 3 treatments: simvastatin 20 mg and placebo, simvastatin 20 mg and losartan 100 mg, or losartan 100 mg and placebo daily during 2 months. This was a randomized, double-blind, placebo-controlled study with 3 treatment arms (each 2 months) and crossover with 2 washout periods (each 2 months). The patients were seen at 14-day intervals (or more

frequently) during the study. Calcium channel or β-adrenergic blockers were withheld for ≥48 hours before the study to avoid the effects of these drugs. The Gil Hospital Institute Review Board approved the study, and all participants gave written, informed consent.

Laboratory Assays

Blood samples for laboratory assays were obtained at approximately 8 AM after patients fasted overnight before and at the end of each 2-month treatment period. These samples were immediately coded so that investigators performing laboratory assays were blinded to subject identity or study sequence. Assays for lipids, glucose, and plasma malondialdehyde (MDA), monocyte chemoattractant protein (MCP)-1, and adiponectin were performed in duplicate by ELISA (BIOXYTECH LPO-586, OxisResearch, and R&D Systems, Inc) and assays for high-sensitivity C-reactive protein (hsCRP) levels by latex agglutination [CRP-Latex(II), Denka-Seiken] as previously described.^{7,8,18,20} Assays for plasma insulin levels were performed in duplicate by immunoradiometric assay (INSULIN-RIA-BEAD II, Abbott Japan). The interassay and intra-assay coefficients of variation were <6%. Quantitative Insulin-Sensitivity Check Index (QUICKI), a surrogate index of insulin sensitivity, was calculated as follows (insulin is expressed in μU/mL and glucose in mg/dL): QUICKI = 1/[log(insulin) + log(glucose)].¹⁹

Vascular Studies

Imaging studies of the right brachial artery were performed with an ATL HDI 3000 ultrasound machine (Bothell) equipped with a 10-MHz linear-array transducer based on a previously published technique.^{7,8,18,20} Measurements were performed by 2 independent investigators (S.H.H. and W.-J.C.) blinded to the subject's identity and medication status. Measurements of maximum diameter and percent flow-mediated dilation were made in 10 studies selected at random. The interobserver and intraobserver variabilities for repeated measurement of maximum diameter were 0.01±0.06 and 0.008±0.05 mm, respectively. The interobserver and intraobserver variabilities for repeated measurement of percent flow-mediated dilation were 0.12±1.31% and 0.10±1.29%, respectively.

Statistical Analysis

Data are expressed as mean±SEM or median (25% to 75% range). After testing data for normality, we used Student's paired *t* or Wilcoxon signed-rank test to compare values before and after each treatment and the relative changes in values in response to treatment, as reported in Tables 2 and 3. The effects of the 3 therapies on vascular function, markers of oxidant stress and inflammation, and insulin sensitivity relative to baseline values were analyzed by 1-way repeated-measures ANOVA or Friedman's repeated ANOVA on ranks. After demonstration of significant differences among therapies by ANOVA, post hoc comparisons between treatment pairs were made by use of the Student-Newman-Keuls multiple comparison procedures. Pearson's correlation coefficient analysis was used to assess associations between measured parameters. We calculated that 30 subjects would provide 80% power for detecting a difference of absolute increase, ≥2.1% flow-mediated dilation of the brachial artery between baseline and simvastatin, with α=0.05 based on our previous studies.^{7,20} The comparison of endothelium-dependent dilation among the 3 treatment schemes was prospectively designated as the primary end point of the study. All other comparisons were considered secondary. A value of *P*<0.05 was considered statistically significant.

Results

When baseline values before each treatment period were compared among the 3 treatment arms, no significant differences were noted in any of the parameters measured (Tables 2 and 3). To rule out the possibility of a carryover effect from one treatment period to the next, we compared baseline values before the first treatment period to those before the

TABLE 2. Effects of Simvastatin, Combined Therapy, and Losartan on Lipid Levels and Endothelial Function in Hypercholesterolemic, Hypertensive Patients

Variables	Simvastatin		Simvastatin + Losartan		Losartan		P			
	Baseline 1	Treatment	Baseline 2	Treatment	Baseline 3	Treatment	ANOVA	S/C	S/L	C/L
Heart rate, bpm	70±2	70±2	73±2	75±2	75±2	77±2	0.206			
Systolic BP, mm Hg	145±2	142±2	147±3	133±3‡	145±2	128±2‡	<0.001	<0.05	<0.05	NS
Diastolic BP, mm Hg	90±1	87±2	91±1	83±2‡	89±1	79±2‡	<0.001	<0.05	<0.05	NS
Lipids, mg/dL										
Total cholesterol	247±5	173±4‡	242±4	172±4‡	238±5	235±4	<0.001	NS	<0.05	<0.05
Triglycerides	172±12	145±10*	179±13	140±11†	186±16	167±13	0.556			
LDL cholesterol	160±5	93±4‡	154±5	93±4‡	149±5	152±4	<0.001	NS	<0.05	<0.05
ApoB	133±3	90±3‡	129±3	91±2‡	128±2	125±2	<0.001	NS	<0.05	<0.05
HDL cholesterol	53±2	50±2	51±2	52±2	52±2	52±2	0.093			
ApoA-I	161±3	183±3	158±3	168±4†	157±3	158±3	0.020	<0.05	NS	<0.05
Vasomotor, %										
Flow-mediated dilation, %	4.88±0.15	6.54±0.12‡	4.79±0.14	7.88±0.16‡	4.75±0.12	6.19±0.19‡	<0.001	<0.05	NS	<0.05
NTG dilation, %	14.07±0.42	14.50±0.49	14.00±0.48	14.67±0.60	14.12±0.51	14.16±0.64	0.575			
MDA, μmol/L	0.98±0.05	0.87±0.05‡	0.93±0.05	0.70±0.06‡	0.98±0.05	0.93±0.05*	<0.001	<0.05	NS	<0.05
Inflammation, mg/L										
CRP	0.85 (0.30–2.70)	0.80 (0.40–1.40)*	0.85 (0.50–2.00)	0.65 (0.30–1.30)†	0.85 (0.50–2.30)	0.80 (0.40–1.50)*	0.146			
MCP-1	190±8	170±6†	198±8	168±6‡	192±9	178±8*	0.030	<0.05	NS	<0.05

S indicates simvastatin; C, combined therapy; L, losartan; BP, blood pressure; Apo, apolipoprotein; and NTG, nitroglycerin. Data are expressed as mean±SEM or median (25th to 75th percentiles). There were no significant differences among each baseline value.

* $P<0.05$, † $P<0.01$, ‡ $P<0.001$ vs each baseline value.

second and third treatment periods. There were no significant differences in any of the measured parameters in this analysis.

Effects of Therapies on Blood Pressure and Lipids

Losartan alone or combined therapy significantly reduced systolic and diastolic blood pressures after 2 months of administration compared with baseline. These reductions were significantly greater than that observed with simvastatin alone ($P<0.001$ by ANOVA). Simvastatin alone or combined therapy significantly lowered total cholesterol (both $P<0.001$), LDL cholesterol (both $P<0.001$), and apolipoprotein B levels (both $P<0.001$) compared with baseline. These reductions were significantly greater than those observed with losartan alone ($P<0.001$ by ANOVA). However, there were no significant differences between simvastatin alone and combined therapy for these parameters (Table 2).

Effects of Therapies on Vasomotor Function and MDA

Simvastatin, combined therapy, and losartan significantly improved the percent flow-mediated dilator response to

hyperemia relative to baseline measurements by 38±4%, 68±4%, and 31±3%, respectively (all $P<0.001$); however, combined therapy significantly improved this response more than simvastatin or losartan alone ($P<0.001$ by ANOVA; Figure 1 and Table 2). The brachial artery dilator response to nitroglycerin was similar for all therapies and was not significantly changed from baseline values. Simvastatin, combined therapy, and losartan significantly decreased the plasma MDA levels relative to baseline measurements by 11±3% ($P<0.001$), 23±4% ($P<0.001$), and 5±3% ($P=0.040$), respectively; however, combined therapy significantly reduced MDA levels more than simvastatin or losartan alone ($P<0.001$ by ANOVA; Figure 2 and Table 2).

Effects of Therapies on Markers of Inflammation

Simvastatin, combined therapy, and losartan significantly decreased plasma MCP-1 levels relative to baseline measurements by 7±3% ($P=0.003$), 15±3% ($P<0.001$), and 5±4% ($P=0.048$), respectively; however, combined therapy significantly decreased MCP-1 levels more than simvastatin or

TABLE 3. Effects of Simvastatin, Combined Therapy, and Losartan on Adiponectin Levels and Insulin Resistance in Hypercholesterolemic, Hypertensive Patients

Variables	Simvastatin		Simvastatin + Losartan		Losartan		P			
	Baseline 1	Treatment	Baseline 2	Treatment	Baseline 3	Treatment	ANOVA	S/C	S/L	C/L
ADP, μg/mL	4.5 (3.4–7.0)	4.5 (2.9–6.0)	4.6 (3.3–6.4)	5.0 (3.0–7.3)‡	4.2 (3.5–6.2)	5.3 (3.8–6.8)†	<0.001	<0.05	<0.05	NS
Insulin, μU/mL	2.67±0.29	3.06±0.38	2.56±0.26	2.40±0.28	2.79±0.26	2.49±0.28	0.041	0.064	<0.05	NS
Glucose, mg/dL	82±2	83±2	82±2	79±2	83±2	80±2	0.348			
QUICKI	0.475±0.016	0.458±0.013	0.467±0.012	0.497±0.014*	0.456±0.012	0.483±0.015*	0.029	0.054	<0.05	NS

S indicates simvastatin; C, combined therapy; L, losartan; and ADP, adiponectin. Data are expressed as mean±SEM or median (25th to 75th percentiles). There were no significant differences among each baseline value.

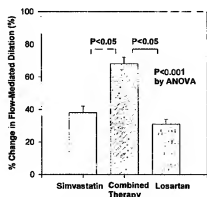


Figure 1. Percent change in flow-mediated dilation from pretreatment values after treatment with simvastatin alone, combined therapy, and losartan alone ($P<0.001$ by ANOVA). Bars indicate SEM.

losartan alone ($P=0.030$ by ANOVA; Figure 3 and Table 2). Simvastatin, combined therapy, and losartan significantly lowered plasma hsCRP levels relative to baseline measurements from 0.85 to 0.80 mg/L ($P=0.042$), 0.85 to 0.65 mg/L ($P=0.002$), and 0.85 to 0.80 mg/L ($P=0.042$), respectively; however, the magnitude of reduction among these 3 therapies was not statistically significant ($P=0.146$ by ANOVA).

Effects of Therapies on Adiponectin and Insulin Resistance

There were significant inverse correlations between body mass index and baseline plasma adiponectin levels ($r=-0.332$, $P=0.023$ before simvastatin; $r=-0.328$, $P=0.024$ before combined therapy; $r=-0.292$, $P=0.046$ before losartan). There were significant inverse correlations between baseline adiponectin levels and baseline triglyceride levels ($r=-0.351$, $P=0.016$ before simvastatin; $r=-0.325$, $P=0.026$ before combined therapy; $r=-0.342$, $P=0.019$ before losartan). There were significant correlations between baseline adiponectin levels and baseline HDL cholesterol levels ($r=0.401$, $P=0.005$ before simvastatin; $r=0.399$, $P=0.006$ before combined therapy; $r=0.303$, $P=0.039$ before losartan). Combined therapy and losartan alone significantly increased the plasma adiponectin levels relative to baseline measurements from 4.63 to 5.02 ($P<0.001$) and 4.19

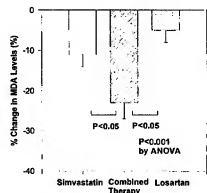


Figure 2. Percent change in MDA levels from pretreatment values after treatment with simvastatin alone, combined therapy, and losartan alone ($P<0.001$ by ANOVA). Bars identify SEM.

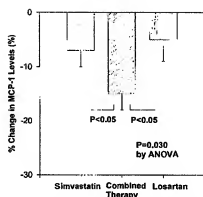


Figure 3. Percent change in MCP-1 levels from pretreatment values after treatment with simvastatin alone, combined therapy, and losartan alone ($P=0.030$ by ANOVA). Bars identify SEM.

to 5.27 ($P=0.002$), respectively. These increases were significantly greater than those observed with simvastatin alone ($P<0.001$ by ANOVA; Figure 4 and Table 3). The 3 therapies did not have significantly different baseline insulin and glucose levels. However, the magnitude of reduction of insulin with combined therapy or losartan alone was significantly greater than with simvastatin alone ($P=0.041$ by ANOVA; Table 3). Combined therapy or losartan alone significantly increased QUICKI relative to baseline measurements by $7\pm3\%$ ($P=0.032$) and $7\pm3\%$ ($P=0.042$), respectively. These increases were significantly greater than those observed with simvastatin alone ($P=0.029$ by ANOVA; Figure 5 and Table 3). There were correlations between percent changes in adiponectin levels and percent changes in QUICKI ($r=0.245$, $P=0.097$ after simvastatin; $r=0.316$, $P=0.030$ after combined therapy; $r=0.433$, $P=0.002$ after losartan). There were inverse correlations between percent changes in adiponectin levels and percent changes in insulin ($r=-0.171$, $P=0.251$ after simvastatin; $r=-0.352$, $P=0.015$ after combined therapy; $r=-0.367$, $P=0.011$ after losartan).

We investigated whether losartan-induced changes in the percent flow-mediated dilator response to hyperemia, serological markers of oxidant stress and inflammation, and insulin resistance were mediated by a reduction in systolic or

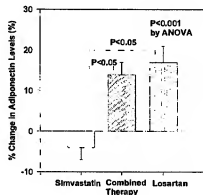


Figure 4. Percent change in adiponectin levels from pretreatment values after treatment with simvastatin alone, combined therapy, and losartan alone ($P<0.001$ by ANOVA). Bars identify SEM.

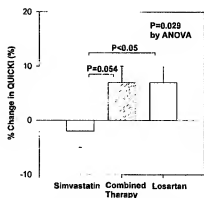


Figure 5. Percent change in QUICKI from pretreatment values after treatment with simvastatin alone, combined therapy, and losartan alone ($P=0.029$ by ANOVA). Bars identify SEM.

diastolic blood pressure. There were no significant correlations between these changes and reduction of systolic blood pressure ($-0.134 \leq r \leq 0.077$) or between these changes and reduction of diastolic blood pressure ($-0.295 \leq r \leq 0.172$). After combined therapy, improvement in flow-mediated dilation correlated with changes in MDA levels ($r=-0.422$ and $P=0.003$), MCP-1 levels ($r=0.189$ and $P=0.204$), hsCRP levels ($r=-0.137$ and $P=0.357$), adiponectin levels ($r=0.420$ and $P=0.003$), QUICKI ($r=0.258$ and $P=0.080$), and insulin levels ($r=-0.251$ and $P=0.089$).

Discussion

In our hypercholesterolemic, hypertensive cohort, simvastatin therapy alone significantly improved the lipid profile, whereas losartan therapy alone significantly lowered blood pressure as expected. Comparable beneficial effects on both lipids and blood pressure were observed with combination therapy. We reasoned that distinct biological actions of simvastatin and losartan therapies on lipoproteins and the angiotensin system may improve endothelium-dependent vascular function by different mechanisms. Indeed, although monotherapy with simvastatin or losartan significantly improved endothelial function and inflammatory markers (assessed by flow-mediated dilation, MDA levels, CRP levels, and MCP-1 levels), combined therapy had additional substantial and significant beneficial effects on these parameters over those seen with monotherapy for either drug, which may explain the observations of a recent clinical trial.²¹

The additional beneficial effects of combined simvastatin/losartan therapy may be the result of several interacting mechanisms. For example, angiotensin II is very potent endogenous vasoconstrictor, whereas LDL induces upregulation of the AT1 receptor.²² Indeed, hypercholesterolemic rabbits display enhanced vascular expression of AT1 receptors that mediate increased activity of angiotensin II.²³ Furthermore, the effect of statins to reverse the elevated blood pressure response to angiotensin II infusion is accompanied by downregulated AT1 receptor density.^{24,25} Angiotensin II promotes superoxide anion generation and endothelial dysfunction.^{8,26} CRP upregulates AT1 receptors in vascular smooth muscle cells, and these effects are attenuated by losartan.²⁷ The additive beneficial effects of combined ther-

apy in the present study are consistent with experimental and clinical studies.^{21,28}

Losartan therapy alone resulted in significant elevation of adiponectin levels, decreased insulin levels, and increased insulin sensitivity (assessed by QUICKI). The present study is the first report demonstrating that losartan therapy can increase adiponectin levels. Adiponectin is an adipose-derived factor that augments and mimics metabolic actions of insulin. Increasing adiponectin levels would be predicted to improve both insulin sensitivity and endothelial function by multiple mechanisms. Regulation of metabolic homeostasis and hemodynamic homeostasis may be coupled by vascular actions of insulin to stimulate production of NO.¹⁶ Thus, improvements in endothelial function may increase insulin sensitivity, whereas increased insulin sensitivity may improve endothelial function.¹² Interestingly, in contrast to the effects of combination therapy on flow-mediated dilation, MDA, CRP, and MCP-1, the beneficial effects of losartan therapy on adiponectin levels, insulin levels, and insulin sensitivity did not increase further with combination therapy. This finding suggests that improving endothelial function *per se* (as reflected by flow-mediated dilation) may not completely explain the effects of losartan or combined therapy to improve insulin sensitivity. In other words, there may be additional mechanisms for losartan or combined therapy to improve insulin sensitivity that are independent of endothelial function, eg, direct effects of losartan on glucose insulin-stimulated glucose uptake or promotion of adipogenic differentiation of preadipocytes²⁹ or induction of peroxisome proliferator-activated receptor- γ activity promoting differentiation in adipocytes.³⁰ Effects of losartan or combined therapy to increase adiponectin levels may in part mediate improved insulin sensitivity, which is supported by the significant correlation shown in the present study. On the other hand, combined therapy may reduce insulin resistance by multiple mechanisms such as lipoprotein changes and reduced oxidant stress that also contribute to NO bioavailability. The effects of losartan or combined therapy on flow-mediated dilation, oxidant stress and inflammation markers, and insulin resistance were independent of blood pressure changes and consistent with recent randomized clinical trials.^{2,31} Likewise, several studies suggest a hypothesis that the effects of AT1 receptor blockers to improve endothelial function are due to other factors in addition to a reduction in blood pressure.^{32,33}

Metabolic syndrome is associated with atherosclerotic disease. Patients with metabolic syndrome make up one of the largest groups of individuals with both hyperlipidemia and hypertension. Obesity is one of the most common causes of cardiovascular disease. In the present study, more than half of the subjects were overweight. We observed that plasma levels of adiponectin were significantly inversely correlated with body mass index. We also observed significant correlations between baseline adiponectin levels and baseline HDL cholesterol or triglyceride levels. Thus, our study may have implications for the treatment of patients with metabolic syndrome.

In summary, our study suggests that combination therapy with simvastatin and losartan has beneficial additive effects

on endothelial function and inflammatory markers. This may be due to combined effects of the respective monotherapies to improve lipid profile, blood pressure, adiponectin levels, and insulin sensitivity. The additive beneficial effects of combined therapy are predicted to reduce cardiovascular events in hypercholesterolemic, hypertensive patients more than monotherapy with either drug alone.

Acknowledgments

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EXHIBIT B



Effect of simvastatin and/or pioglitazone on insulin resistance, insulin secretion, adiponectin, and proinsulin levels in nondiabetic patients at cardiovascular risk—the PIOSTAT Study

Thomas Forst^{a,b,*}, Andreas Pfützner^a, Georg Lübben^c, Matthias Weber^b, Nikolaus Marx^d, Efstrathios Karagiannis^c, Carsta Koehler^c, Werner Baurecht^f, Cloth Hohberg^a, Markolf Hanefeld^e

^aInstitute for Clinical Research and Development, Medical Department, D-55116 Mainz, Germany

^bDepartment of Endocrinology, Johannes Gutenberg University, D-55130 Mainz, Germany

^cTAKEDA Pharma GmbH Medical, D-52066 Aachen, Germany

^dDepartment of Cardiology, University Ulm, D-89081 Ulm, Germany

^eGWT-TUD, D-01307 Dresden, Germany

^fAcromion, D-50226 Frechen, Germany

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Abstract

We investigated the effect of pioglitazone in comparison with and in combination with simvastatin on insulin resistance, plasma adiponectin, postprandial plasma glucose, insulin, and intact proinsulin levels in a nondiabetic population at cardiovascular risk. One hundred twenty-five nondiabetic patients at cardiovascular risk were randomized to pioglitazone (PIO), pioglitazone and simvastatin (PIO/SIM), or simvastatin (SIM) treatments. Blood samples were taken for the measurement of adiponectin and lipid levels. In addition, an oral glucose load with the measurements of glucose, insulin, and intact proinsulin levels was performed. Adiponectin levels increased from 14.0 ± 8.2 to 27.6 ± 14.5 $\mu\text{g/mL}$ ($P < .0001$) during PIO treatment and from 11.7 ± 10.0 to 26.7 ± 15.7 $\mu\text{g/mL}$ ($P < .0001$) during PIO/SIM treatment. A decrease in adiponectin levels from 15.5 ± 12.7 to 11.6 ± 7.0 $\mu\text{g/mL}$ ($P < .05$) was observed during SIM treatment. Although fasting intact proinsulin levels remained unchanged, the increase in postprandial intact proinsulin levels could be reduced from 29.5 ± 21.4 to 22.1 ± 17.5 pmol/L ($P < .01$) during PIO treatment and from 24.3 ± 27.4 to 21.1 ± 16.5 mmol/L ($P < .05$) during PIO/SIM treatment. Lipid parameters improved during SIM treatment but not during PIO treatment. Combined treatment with PIO/SIM was superior in improving overall cardiovascular risk profile than every single drug.

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1. Introduction

Several studies have shown a significant impact on glucose metabolism and insulin resistance or coronary heart disease even in patients without diabetes [1–7]. Two laboratory parameters, adiponectin and intact proinsulin, have received increasing attention for the characterization of insulin resistance, beta-cell function, and cardiovascular risk [8–11]. Increased intact proinsulin levels and reduced adiponectin plasma levels were shown to be highly

predictive for cardiovascular disease and type 2 diabetes mellitus [12–15].

Recently, new drugs for intervention of insulin resistance, the peroxisome proliferator-activated receptors (PPARs), were introduced in the treatment of patients with type 2 diabetes mellitus. Beyond their effects on insulin resistance and glucose metabolism, peroxisome proliferator-activated receptor γ (PPAR γ) agonists were shown to interact with several cardiovascular risk factors such as dyslipidemia, intact proinsulin, or adiponectin levels in patients with diabetes and to reduce the risk for myocardial infarction and stroke [16–18]. On the other hand, the most common and clinically applied drug intervention in the treatment of patients with increased cardiovascular risk with or without diabetes is the inhibition of 3-hydroxy-3-methylglutaryl

* Corresponding author. Institute for Clinical Research and Development, Parcussstrasse 8, D-55116 Mainz, Germany. Tel.: +49 6131 5763613; fax: +49 6131 5763611.

E-mail address: thomasf@ikfe.de (T. Forst).

coenzyme A reductase by statins (3-hydroxy-3-methylglutaryl coenzyme A inhibitors) [19,20].

Up to now, no information is available about the clinical effects of PPAR γ stimulation in comparison with or in combination with statins on the previously described metabolic predictors of cardiovascular risk in nondiabetic patients. The aim of this investigation was to evaluate the effects of pioglitazone in comparison with simvastatin on adiponectin, proinsulin, and postload glucose metabolism in nondiabetic patients with increased risk for cardiovascular disease.

2. Patients and methods

This study was a 2-center, prospective, double-blinded, double-dummy, 3-arm parallel trial evaluating the effects of pioglitazone in comparison with and in combination with simvastatin on insulin resistance and adiponectin plasma levels, as well as on fasting and postload glucose, insulin, and proinsulin levels in nondiabetic patients with elevated cardiovascular risk. The study was performed according to the Declaration of Helsinki and Good Clinical Practice; it was approved by the local ethical review board.

2.1. Patients

One hundred thirty-five patients with increased cardiovascular risk, as defined by previous medical history of infarction, and/or coronary angiography with proven cardiovascular disease, and/or unstable angina pectoris, and/or duplex sonography of cervical or leg vessels with proven arteriosclerotic alterations, and/or electrocardiogram with ischemia, and/or stroke, and/or transient ischemic attack, and/or peripheral arterial occlusion, and/or vessel surgery, and/or hypertension were selected for the study. Patients with previous statin treatment and/or PPAR γ -activator treatment within the last 4 weeks before entering the trial were excluded. All patients provided their written informed consent. According to the inclusion criteria, 135 patients were randomized, 132 patients received at least 1 medication, and 125 patients were followed up for at least 1 follow-up investigation and were available for the efficacy analysis. In a double-blinded, double-dummy technique, patients were randomly assigned to 1 of 3 treatment groups: pioglitazone in combination with placebo, pioglitazone in combination with simvastatin, or simvastatin in combination with placebo. Treatment with study medication was started with 30 mg pioglitazone and/or 20 mg simvastatin, and after 2 weeks, the dosage was increased to 45 mg pioglitazone and/or 40 mg simvastatin. The clinical characteristics and previous treatment of the study participants in the different treatment groups are given in Table 1. Even if slightly more patients in the combined pioglitazone and simvastatin group received β -blockers, no statistically significant difference in any of these parameters were evident among the groups. All concomitant medication was kept constant for the observational period.

At baseline and after 3 months of study treatment, blood was taken for the measurement of glucose, insulin, intact

Table 1

Clinical characteristics of the investigated groups

	Pioglitazone (n = 39)	Simvastatin (n = 43)	Simvastatin and pioglitazone (n = 43)
Age (y)	59.5 \pm 7.8	57.3 \pm 8.4	59.0 \pm 8.6
Body mass index (kg/m ²)	30.8 \pm 4.8	30.5 \pm 3.7	31.2 \pm 4.1
Male/female	13/26	16/27	18/25
Hypertension	92.3	90.7	90.7
RAS inhibition therapy	59.0	48.8	62.8
β -Blocking therapy	30.8	34.9	46.5
Calcium channel blockers	17.9	23.3	16.3
Diuretics	12.8	14.0	18.6

Values are expressed as percentages or mean \pm SD; there were no significant differences among the groups. RAS indicates renin angiotensin system inhibition, angiotensin-converting enzyme inhibitors, or angiotensin receptor blockers.

proinsulin, adiponectin, and lipid parameters. Insulin resistance was calculated using the homeostasis model assessment HOMA score (fasting serum insulin [μ U/mL] \times fasting plasma glucose [mmol/L]/22.5). Patients with a calculated HOMA score of 2 or higher (>75th percentile) were considered to be insulin resistant [21]. All measurements were obtained in the morning with patients fasting from midnight onward. Patients were asked to refrain from having coffee and tea for at least 8 hours before each study visit.

In addition, at baseline and after 3 months of treatment, all patients received a standardized oral glucose tolerance test (75 mg glucose in 300 mL solution, Dextro OGT, Hoffmann La Roche, Grenzach, Germany). At baseline and after 30, 60, 90, and 120 minutes, blood was taken for the measurement of insulin and blood glucose concentrations. In addition, intact proinsulin plasma levels were measured at baseline and after 30 and 120 minutes.

Blood pressure was measured with the patient in a seated position for at least 5 minutes with an appropriate cuff on the left arm. Patient height was measured at the first visit, and patient weight and waist-to-hip ratio were determined at baseline and at the end of the study.

2.2. Biochemical parameters

All laboratory measurements for both study sites were analyzed in a central laboratory. Specific insulin levels were measured using an enzyme-linked immunosorbent assay technique (Anthos Mikrosysteme, Krefeld, Germany). Plasma glucose was measured with the glucokinase-dehydrogenase method, cholesterol with the cholesterol oxidase phenol 4 aminoantipyrine peroxidase method, high-density lipoprotein (HDL) cholesterol with the precipitation method, and triglycerides with the glycerolphosphate oxidase phenol 4 aminoantipyrine peroxidase method (DiaSys, Holzheim, Germany). Adiponectin and intact proinsulin (Linco Res, St Charles, MO) concentrations were measured using specific immunoassays.

2.3. Statistical analysis

Data are presented as arithmetic mean \pm SD for continuous variables and as the number/proportion of

Table 2

Laboratory parameters and blood pressure at baseline and end point for the different treatment groups (values are expressed as arithmetic mean \pm SD)

	Pioglitazone		Simvastatin		Pioglitazone and simvastatin	
	Baseline	12 wk	Baseline	12 wk	Baseline	12 wk
Glucose (mmol/L)	5.63 \pm 0.54	5.23 \pm 0.51 ^{*†‡}	5.60 \pm 0.62	5.56 \pm 0.55 [†]	5.70 \pm 0.66	5.50 \pm 0.70 ^{*†}
Insulin (mU/mL)	12.8 \pm 7.4	10.2 \pm 3.6 ^{*†}	13.8 \pm 6.3	14.7 \pm 6.5 ^{†‡}	14.8 \pm 7.1	11.5 \pm 4.5 ^{*‡}
HOMA (mU \cdot mol/mL ²)	3.27 \pm 2.21	2.40 \pm 0.97 ^{*†}	3.52 \pm 2.07	3.63 \pm 1.60 ^{†‡}	3.70 \pm 1.98	2.81 \pm 1.15 ^{*‡}
Adiponectin (μ g/mL)	13.96 \pm 8.16	27.64 \pm 14.49 ^{*†}	15.49 \pm 12.66	11.59 \pm 7.03 ^{*†‡}	11.68 \pm 9.96	26.67 \pm 15.73 ^{*‡}
Total cholesterol (mmol/L)	5.60 \pm 0.99	5.67 \pm 1.17 ^{†‡}	5.73 \pm 1.10	4.44 \pm 0.96 ^{*†}	5.67 \pm 1.26	4.43 \pm 0.95 ^{*†}
HDL cholesterol (mmol/L)	1.41 \pm 0.41	1.44 \pm 0.42	1.43 \pm 0.41	1.52 \pm 0.42 [*]	1.44 \pm 0.45	1.53 \pm 0.45 [*]
LDL cholesterol (mmol/L)	3.50 \pm 0.94	3.56 \pm 1.04 ^{†‡}	3.60 \pm 1.01	2.32 \pm 0.88 ^{*†}	3.68 \pm 1.10	2.40 \pm 0.91 ^{*†}
Triglycerides (mmol/L)	1.50 \pm 0.73	1.46 \pm 0.64 [†]	1.63 \pm 1.64	1.36 \pm 1.16	1.45 \pm 0.59	1.13 \pm 0.39 ^{*†}
ASAT (μ mol/L)	0.43 \pm 0.12	0.39 \pm 0.10	0.44 \pm 0.14	0.44 \pm 0.14	0.46 \pm 0.12	0.43 \pm 0.11
ALAT (μ mol/L)	0.43 \pm 0.23	0.40 \pm 0.18	0.48 \pm 0.25	0.46 \pm 0.20	0.46 \pm 0.22	0.39 \pm 0.16
RR syst (mm Hg)	145 \pm 15	139 \pm 17 [*]	145 \pm 18	140 \pm 13	145 \pm 20	136 \pm 16 [*]
RR diast (mm Hg)	89 \pm 10	85 \pm 12 [*]	90 \pm 11	88 \pm 10 [§]	87 \pm 9	81 \pm 8 ^{*‡}

LDL indicates low-density lipoprotein; ASAT, aspartate aminotransferase; ALT, alanin aminotransferase; RR syst, systolic blood pressure; RR diast, diastolic blood pressure.

^{*} $P < .05$ vs baseline (paired t test).

[†] $P < .05$, pioglitazone vs simvastatin.

[‡] $P < .05$, pioglitazone vs simvastatin and pioglitazone.

[§] $P < .05$, simvastatin vs simvastatin and pioglitazone (2-sample t tests).

patients with a characteristic for categorical variables. The analysis of safety is based on the intention-to-treat population, which consists of all patients who received at least 1 dose of medication. For efficacy analysis, all patients who underwent baseline assessment and at least 1 examination thereafter were included. All analyses were performed in an exploratory sense, and a P value of less than .05 was considered clinically significant.

Treatment groups were compared at baseline by using the Student t test for continuous variables and the χ^2 test for categorical variables. Changes from baseline for distinct cardiovascular risk parameters were evaluated using Student t tests. Within-group comparisons were analyzed by paired t tests, and between-group comparisons were analyzed by 2-sample t tests. The change from baseline was defined as the absolute change from baseline (actual value minus baseline value) and as the relative change from baseline (actual value minus baseline divided by baseline). Correlation was assessed by using the Spearman rank-order correlation coefficient. Statistical analysis was performed using SAS version 8.2 (SAS Institute, Cary, NC).

3. Results

At baseline, mean fasting blood glucose of the entire study group was 5.64 ± 0.61 mmol/L. According to the oral glucose tolerance test, 16.8% of the patients were found to have an impaired glucose tolerance, and 86.4% of the patients had an impaired insulin sensitivity as defined by a HOMA score of greater than 2. Adiponectin levels at baseline were 13.7 ± 10.5 μ g/mL, and an inverse correlation could be obtained between adiponectin plasma levels and insulin resistance (HOMA score: $r = -0.55$, $P < .0001$).

As shown in Table 2, treatment with pioglitazone and the combined treatment with pioglitazone and simvastatin

resulted in a reduction of fasting insulin and glucose levels. Fasting insulin levels decreased significantly during pioglitazone treatment by $-13\% \pm 28\%$ and during combined treatment by $-16\% \pm 26\%$. The HOMA-S score declined by $19\% \pm 28\%$ during pioglitazone monotherapy and by $17\% \pm 29\%$ in the combined-treatment group. No significant change in these parameters could be observed with single simvastatin treatment. The improvement in insulin sensitivity was found to correlate with the reduction in fasting glucose levels ($r = 0.52$, $P < .0001$) and with the improvement in blood glucose levels 2 hours after the oral glucose load ($r = 0.26$, $P = .0045$).

As shown in Fig. 1, adiponectin levels increased by $127\% \pm 105\%$ during pioglitazone monotherapy and by $166\% \pm 94\%$ during treatment with pioglitazone in

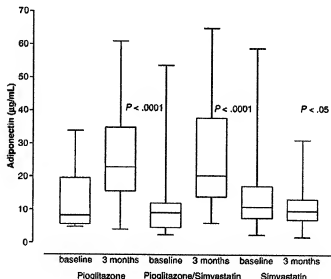


Fig. 1. Adiponectin plasma levels at baseline and after 12 weeks of treatment with pioglitazone, pioglitazone and simvastatin, or simvastatin.

combination with simvastatin. Surprisingly, treatment with simvastatin alone resulted in a small but significant reduction in adiponectin levels by $12\% \pm 30\%$. An inverse correlation was obtained between the improvement in HOMA-S score and the change in adiponectin plasma levels ($r = -0.32$, $P = .0004$). After 3 months of treatment, adiponectin levels were significantly higher in both pioglitazone-treated groups compared with the simvastatin group.

Pioglitazone treatment and the combined treatment were found to significantly improve glucose and insulin profile during the oral glucose tolerance test, whereas no significant effect could be observed during simvastatin monotherapy. As illustrated in Fig. 2A to C, fasting intact proinsulin levels were found in the upper normal limit. After the glucose load, a pronounced increase in intact proinsulin levels could be observed in all treatment groups. Although no significant change in fasting intact proinsulin levels could be observed in any treatment group, pioglitazone and the combination of pioglitazone with simvastatin significantly reduced the increase in postprandial intact proinsulin levels.

As shown in Table 2, single treatment with simvastatin and the combined treatment with simvastatin and pioglitazone resulted in a significant improvement in total cholesterol, HDL, and low-density lipoprotein levels.

A significant decrease in systolic and diastolic blood pressures could be observed in pioglitazone-treated patients and in the group receiving both study drugs, whereas only a nonsignificant decrease in blood pressure could be observed with single simvastatin treatment.

3.1. Safety and tolerability

All 3 kinds of treatment were generally well tolerated. There was no significant difference in the number of adverse events among the study groups. Treatment with pioglitazone was associated with a higher number of peripheral edema in

the pioglitazone monotherapy (11.4%) and in the combined-treatment group (22.2%) compared with the simvastatin monotherapy group (7.0%). There was 1 serious adverse event in the pioglitazone monotherapy group, where a patient needed to be hospitalized because of nephrolithiasis.

As shown in Table 2, no adverse effect could be found on liver function in any of the observed groups.

Body weight increased significantly in the pioglitazone-treated (1.8 ± 3.6 kg, $P = .003$) and the combination-treated patients (1.2 ± 2.2 kg, $P < .001$). No significant changes could be observed in the waist-to-hip ratio.

4. Discussion

In our study, 16.8% of nondiabetic patients with increased cardiovascular risk were found with impaired glucose tolerance, and 86.4% of the patients were identified with an impaired insulin sensitivity according to a HOMA-S score of higher than 2. None of the included patients had diabetes according to the definition of the American Diabetes Association.

Consistent with previous studies showing a strong effect of pioglitazone treatment on adiponectin plasma levels in diabetic patients [22,23], our study revealed a striking improvement in adiponectin levels also in nondiabetic patients at cardiovascular risk. Besides increasing adiponectin levels, PPAR α and PPAR γ stimulations were shown to increase the expression of adiponectin receptors in macrophages and might therefore evolve substantial therapeutic effects in the development and progression of arteriosclerosis [24]. Treatment with pioglitazone or the combined treatment with pioglitazone and simvastatin for 3 months resulted in a 127% and 166% increase in adiponectin plasma levels, respectively. In contrast to the results obtained by Chu et al [25] in patients with type 2

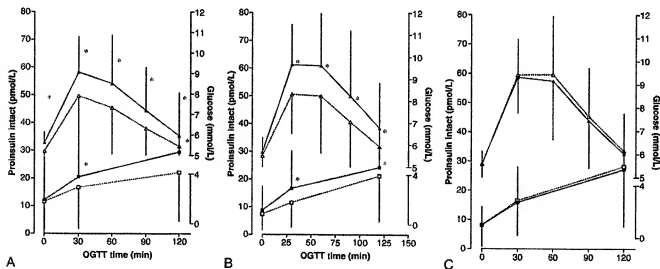


Fig. 2. Time course of postprandial glucose and proinsulin intact plasma levels at baseline and 12 weeks after treatment with pioglitazone (A), pioglitazone and simvastatin (B), or simvastatin (C). Values are expressed as mean \pm SD. ■ indicates proinsulin at baseline; □, proinsulin after 12 weeks of treatment; ▲, glucose at baseline; △, glucose after 12 weeks of treatment; OGTT, oral glucose tolerance trial. * $P < .001$.

diabetes mellitus, in our study, single treatment with simvastatin slightly, but significantly, reduced adiponectin plasma levels. The reason for this discrepancy might be caused by the different patient populations investigated in the 2 studies (diabetic vs nondiabetic patients).

In our study, treatment with pioglitazone and the combination of pioglitazone with simvastatin significantly improved the time course of glucose and insulin levels after an oral glucose load.

Intact proinsulin plasma concentrations were found to be elevated in obese patients and in patients with insulin resistance and were shown to be a strong predictor for type 2 diabetes mellitus and cardiovascular disease [8,26–28]. In addition, an association between increased proinsulin levels and a sympathoadrenal imbalance of the autonomic nervous system is described for diabetic and nondiabetic subjects [29]. Heretofore, nothing is known about the release of intact proinsulin in the postprandial state in nondiabetic patients at increased cardiovascular risk. In contrast to the only moderately elevated intact proinsulin levels at baseline, a remarkable increase in intact proinsulin levels could be observed after the oral glucose load. Treatment with the PPAR γ agonist pioglitazone in patients with type 2 diabetes mellitus were shown to significantly reduce intact proinsulin levels [30], which was found to be associated with an improvement in other cardiovascular risk markers such as intima-media thickness of the carotid artery [31]. In our recent study, treatment with pioglitazone and the combination of pioglitazone with simvastatin did not affect fasting intact proinsulin levels but significantly decreased the increase in postprandial intact proinsulin levels. No effect of simvastatin monotherapy on intact proinsulin levels could be observed in this patient population. The finding of a considerable increase of intact proinsulin levels in the postprandial state is new, and might have important clinical implications. Pursuing research has to clarify the role of the postprandial increase in intact proinsulin levels as an independent cardiovascular risk factor in patients with insulin resistance.

In our study, treatment with simvastatin and the combination of simvastatin and pioglitazone significantly improved total cholesterol, HDL, and low-density lipoprotein plasma levels. In contrast to previous studies on diabetic patients [32], no significant effect on these lipid parameters could be observed during treatment with pioglitazone alone.

In conclusion, this is the first study that investigated the effect of pioglitazone in comparison with and in combination with simvastatin on several markers of the metabolic syndrome in nondiabetic patients with increased cardiovascular risk. Despite the specific beneficial action of each single drug on distinct cardiovascular risk factors, the combined treatment with both study drugs was able to provide an overall improvement of all risk markers observed in our study. The results of the study also prove additional protective effects of PPAR γ agonists on the cardiovascular system in patients pretreated with statins. Further studies need to evaluate the clinical significance of these beneficial

effects of combining PPAR γ agonist and statin treatments in reducing the cardiovascular risk in patients without diabetes.

Acknowledgment

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Appendix A

This study was performed at 2 sites:

1. GWT-TUD, Center for Clinical Studies, Dresden, Germany
2. Institute for Clinical Research and Development, Mainz, Germany.

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EXHIBIT C

Circulating Adiponectin and Resistin Levels in Relation to Metabolic Factors, Inflammatory Markers, and Vascular Reactivity in Diabetic Patients and Subjects at Risk for Diabetes

GREESHMA K. SHETTY, MD¹
PANAYIOTIS A. ECONOMIDES, MD^{1,2,3}
EDWARD S. HORTON, MD^{1,3}

CHRISTOS S. MANTZOROS, MD¹
ARISTIDIS VEVES, MD^{1,2}

OBJECTIVE — Adiponectin and resistin, two recently discovered adipocyte-secreted hormones, may link obesity with insulin resistance and/or metabolic and cardiovascular risk factors. We performed a cross-sectional study to investigate the association of adiponectin and resistin with inflammatory markers, hyperlipidemia, and vascular reactivity and an interventional study to investigate whether atorvastatin mediates its beneficial effects by altering adiponectin or resistin levels.

RESEARCH DESIGN AND METHODS — Associations among vascular reactivity, inflammatory markers, resistin, and adiponectin were assessed cross-sectionally using fasting blood samples obtained from 77 subjects who had diabetes or were at high risk to develop diabetes. The effect of atorvastatin on adiponectin and resistin levels was investigated in a 12-week-long randomized, double-blind, placebo-controlled study.

RESULTS — In the cross-sectional study, we confirm prior positive correlations of adiponectin with HDL and negative correlations with BMI, triglycerides, C-reactive protein (CRP), and plasma activator inhibitor (PAI)-1 and report a negative correlation with tissue plasminogen activator. The positive association with HDL and the negative association with PAI-1 remained significant after adjusting for sex and BMI. We also confirm prior findings of a negative correlation of resistin with HDL and report for the first time a positive correlation with CRP. All of these associations remained significant after adjusting for sex and BMI. No associations of adiponectin or resistin with any aspects of vascular reactivity were detected. In the interventional study, atorvastatin decreased lipid and CRP levels, but adiponectin and resistin were not specifically altered.

CONCLUSIONS — We conclude that adiponectin is significantly associated with inflammatory markers, in part, through an underlying association with obesity, whereas resistin's associations with inflammatory markers appear to be independent of BMI. Lipid profile and inflammatory marker changes produced by atorvastatin cannot be attributed to changes of either adiponectin or resistin.

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From the ¹Division of Endocrinology, Diabetes and Metabolism, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts; the ²Microcirculation Laboratory, Beth Israel Deaconess Medical Center West Campus, Harvard Medical School, Boston, Massachusetts; and the ³Joslin Diabetes Center, Harvard Medical School, Boston, Massachusetts.

Address correspondence and reprint requests to Christos S. Mantzoros, MD, Division of Endocrinology, Diabetes and Metabolism, Department of Internal Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, 99 Brookline Ave., RN 325A, Boston, MA 02215. E-mail: cmantzor@bidmc.harvard.edu.

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Abbreviations: ACH%, acetylcholine chloride with percentage increase over baseline of endothelium-dependent vasodilation; CRP, C-reactive protein; ET1, endothelin-1; ICAM, intracellular adhesion molecule; NID, nitroglycerin-induced dilation; NNP%, sodium nitroprusside with percentage increase over baseline of endothelium-independent vasodilation; PAI, plasma activator inhibitor; TNF- α , tumor necrosis factor- α ; tPA, tissue plasminogen activator.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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Obesity and insulin resistance are associated with cardiovascular risk factors, including altered levels of inflammatory markers and endothelial dysfunction (1). Two recently identified adipocyte-secreted hormones, adiponectin and resistin, are thought to link obesity with insulin resistance and cardiovascular risk (1–7). Adiponectin, a recently discovered 244-amino acid, adipose-specific protein (2), is found in high concentrations in the peripheral circulation (3), and its circulating levels are decreased in obesity and type 2 diabetes (4). Adiponectin levels are inversely associated with central or overall adiposity, as well as hyperlipidemia and insulin resistance independently of BMI (5,6). Resistin levels have been reported to be markedly elevated in obese mice and to be decreased by insulin sensitizers, such as rosiglitazone and other thiazolidinediones, and by the administration of antiresistin antiserum, which also leads to a significant decrease in blood glucose (7). These initial findings in mice were challenged by subsequent observations, however, demonstrating increased or decreased resistin levels in obesity as well as variable responses to thiazolidinediones (8,9) and a null association between circulating resistin and insulin resistance in humans (10,11). Interestingly, although resistin has structural similarities to proteins involved in inflammatory processes (12), circulating resistin levels have never been studied in relation to inflammatory markers in humans. In contrast, adiponectin has been associated with markers of inflammation, such as C-reactive protein (CRP) and tumor necrosis factor- α (TNF- α) (13,14), but associations with several other inflammatory markers remain to be studied.

Thus, we have studied cross-sectionally whether adiponectin is inversely associated and if resistin is

positively associated with inflammatory markers, hyperlipidemia, and vascular reactivity. We also studied, in the context of a 12-week-long, double-blind, randomized, placebo-controlled clinical trial, whether atorvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, which reduces total cholesterol, LDL (15), and triglycerides (16), increases HDL levels (17), and may mediate improved insulin sensitivity (18), affects these outcomes by altering adiponectin and/or resistin levels.

RESEARCH DESIGN AND METHODS

The present study included 77 previously studied subjects (19) who had diabetes with no serious long-term complications (defined as macroalbuminuria, severe neuropathy, and/or peripheral vascular disease associated with foot ulceration or other lower-extremity amputations) or were considered at higher risk to develop type 2 diabetes than the general population (a positive family history and a normal 75-g oral glucose tolerance test, subjects with at least one first-degree relative with type 2 diabetes, or subjects with impaired glucose tolerance), using previously described criteria (20). The study protocol was approved by the institutional review boards at the Joslin Diabetes Center and the Beth Israel Deaconess Medical Center. After being informed on the purpose and procedures of the study, all subjects signed an informed consent form. Subjects were evaluated at an initial screening visit, which involved a full medical history and physical examination including blood pressure, weight, height, waist-to-hip ratio, funduscopy, and evaluation for clinical signs of neuropathy. Subjects were excluded if they had a known history of cardiovascular disease, stroke or transient ischemic attack, uncontrolled hypertension, liver disease, renal disease, severe dyslipidemia (triglycerides >600 mg/dl or cholesterol >350 mg/dl), or any other serious chronic disease requiring active treatment. Women of child-bearing potential not using an effective form of nonhormonal birth control and subjects taking lipid-lowering agents during the last 3 months, glucocorticoids, antineoplastic agents, psychoactive agents, or bronchodilators on a regular basis were also excluded.

Cross-sectional study

Blood samples were obtained after an overnight fast (~10 h) and a 24-h period free of alcohol or vigorous exercise. Subjects were asked not to take their diabetes medications (sulfonylureas or metformin) for 12 h before each visit. Those subjects using insulin were requested to hold the rapid-acting insulin the morning of each visit. Blood samples were drawn from an antecubital vein with a 19-gauge needle without venous stasis, and the sera were stored at -70°C .

Interventional study

The effect of atorvastatin on adiponectin and resistin levels was investigated in a randomized, double-blind, placebo-controlled study. The 77 subjects described above in the cross-sectional study were randomized in a double-blind manner to receive either 20 mg of atorvastatin daily or the matching placebo for 12 weeks. The subjects were asked to continue with their prior medications, diet plan, and physical activity level in a constant manner for the duration of the study. Subjects were evaluated at baseline and reevaluated after a 12-week treatment period at an exit visit with repeat laboratory evaluations. Vascular reactivity testing was also done at baseline and repeated at 12 weeks. To evaluate the association of serum adiponectin levels, serum resistin levels, and markers of vascular inflammation, fasting blood samples were obtained at baseline and at 12 weeks.

Hormone measurements

Blood samples were analyzed for HDL cholesterol, LDL cholesterol, total cholesterol, triglycerides, endothelin-1 (ET1), CRP, tumor necrosis factor- α (TNF- α), intracellular adhesion molecule (ICAM), plasma activator inhibitor (PAI-1), tissue plasminogen activator (tPA), adiponectin, and resistin. Plasma total cholesterol, HDL cholesterol, and triglycerides were measured using the Synchro CX analyzer (Beckman/Coulter) as previously described (21). LDL cholesterol was calculated from these results. Hormone concentrations were measured using commercially available methods (10) as follows: adiponectin radioimmunoassay (Linco Research, St. Charles, MO) (sensitivity 2 $\mu\text{g}/\text{ml}$; intra-assay coefficient of variation [CV] 1.78–6.21%), human resistin (BioVendor, Brno, Czech Republic) (0.2 ng/ml; 3.4–5.2%), soluble ICAM (R

& D Systems, Minneapolis, MN) (0.35 ng/ml; 3.3–4.8%), and ET1 (R & D Systems) (1.0 pg/ml; 4.2–4.6%) were measured in plasma by the enzyme-linked immunosorbent assay method. TNF- α (Immuntite DPC) (8.1 pg/ml; 2.6–3.6%) and CRP (Immuntite DPC) (0.01 mg/dl; 4.2–6.4%) were measured by chemiluminescent immunoassay. PAI-1 (Diagnostica Stago, France) (1.0 ng/ml; 5.48–6.53%) and tPA (Diagnostica Stago) (1.0 ng/ml; 4.49–4.54%) were also measured by the enzyme-linked immunosorbent assay method. To minimize variability, hormone levels were measured in one assay for all subjects participating in the cross-sectional study and in one assay for each subject in the interventional studies.

Vascular reactivity tests

The vascular reactivity of the forearm skin microcirculation was evaluated by laser Doppler perfusion imaging measurements before and after iontophoresis of acetylcholine chloride [AChCl], percent increase over baseline of endothelium-dependent vasodilation) and sodium nitroprusside (NNP%), percent increase over baseline of endothelium-independent vasodilation) and as previously described (22). To assess the endothelium-dependent reactivity in the macrocirculation, the flow-mediated brachial artery dilation was measured by using a high-resolution ultrasound with a 10.0-MHz linear array transducer and an HDI Ultramark 9 system (Advanced Technology Laboratories, Bothell, WA). Reactive hyperemia was produced by inflating a pneumatic tourniquet to 50 mmHg above the systolic pressure for 5 min distal to the brachial artery, and the flow at rest and reactive flow were measured. All measurements were in accordance with recently published guidelines (23). Endothelium-independent vasodilation in the macrocirculation (nitroglycerin-induced dilation [NID]) was assessed by studying brachial artery diameter changes 5 min after the administration of 400 μg of sublingual nitroglycerin (19). This test was performed 15 min after the reactive hyperemia test and after obtaining a new baseline reading.

Data analysis

Data were summarized using standard procedures. Any missing values due to insufficient sample or values outside the assay range were excluded from the analysis.

Table 1—Baseline characteristics of the subjects

	At risk of type 2 diabetes	Diabetic patients
Total patients	37	40
Age (years)	49 \pm 12	53 \pm 13
Men	20 (54)	23 (58)
With first-degree relative (normal glucose tolerance)	25 (68)	—
With impaired glucose tolerance	12 (32)	—
Type 1/type 2 diabetes	—	20/20
Diabetes duration (years)	—	8 \pm 12
BMI (kg/m ²)	29.5 \pm 5.8	29.8 \pm 9.4
Fasting glucose (mg/dl)	90 \pm 11	178 \pm 79
HbA _{1c} (%)	5.4 \pm 0.4	8.0 \pm 1.6
Total cholesterol (mg/dl)	206 \pm 40	205 \pm 42
LDL (mg/dl)	125 \pm 31	124 \pm 35
HDL (mg/dl)	59 \pm 19	60 \pm 14
Triglycerides (mg/dl)	118 \pm 78	102 \pm 75
Diabetes treatment		
Diet	—	1 (3)
Oral agents	—	15 (37)
Insulin	—	24 (60)
Resting brachial artery diameter (mm)	3.4 \pm 0.7	3.6 \pm 0.7
FMD	5.8 (4.0–9.3)	5.0 (3.2–7.8)
NID	15.2 (11.4–20.9)	13.4 (10.1–17.1)
ACH%	158 \pm 77	149 \pm 77
NNP%	88 \pm 49	93 \pm 44

Data are mean \pm SD, n (%), or median (25–75 percentiles). FMD, flow-mediated dilation percentage increase over baseline (endothelium-dependent reactivity of macrocirculation).

of this specific variable only. In the cross-sectional study, we performed both Spearman's and Pearson's correlation analysis, and we report herein the Spearman's correlation coefficients that are more conservative. Then we performed bivariate and multivariate regression analyses. We evaluated for potential associations among serum adiponectin levels and serum resistin levels (dependent variables) and BMI, metabolic markers (lipid profile), inflammatory parameters (ICAM, ET1, TNF- α , CRP, PAI-1, tPA), as well as vascular reactivity, all expressed as continuous variables. In the multivariate analysis models, we evaluated the same independent variables after controlling for potential confounding by sex, sex and BMI, and sex, BMI, and HbA_{1c}. Logarithmic transformation was used to normalize nonnormally distributed dependent variables.

In the interventional study, we assessed the differences in adiponectin and resistin levels, lipid levels and inflammatory marker levels, as well as BMI using a paired *t* test for parametrically distributed data and the Wilcoxon matched-pair

signed-rank test for nonparametrically distributed data to compare baseline data and data at the end of the study. The *t* test was used to compare the baseline characteristics between those receiving active treatments and those receiving placebo.

SPSS 8.0 software (SPSS, Chicago, IL) was used for statistical analysis. A *P* value <0.025 (two tailed) was considered statistically significant for all analyses (Bonferroni correction). Descriptive statistics are presented as mean \pm SE.

RESULTS

Cross-sectional study

Baseline characteristics of the study population are presented in Table 1. Spearman correlations, including all subjects, among adiponectin and the metabolic markers, inflammatory markers, and vascular reactivity, confirmed prior findings of the positive correlation with HDL ($r = 0.42$, $P < 0.01$) and the negative correlations with triglycerides ($r = -0.35$, $P < 0.01$), CRP ($r = -0.21$, $P = 0.026$), PAI-1 ($r = -0.39$, $P < 0.01$), and BMI ($r = -0.32$, $P < 0.01$). Additionally, we

discovered the negative correlation of adiponectin with tPA ($r = -0.30$, $P < 0.01$) (Table 2). We found no correlation of adiponectin with vascular reactivity or glucose levels. Spearman correlations, including all subjects, among resistin and the metabolic markers, inflammatory markers, and vascular reactivity, confirmed prior findings of a negative correlation with HDL ($r = -0.23$, $P < 0.01$) and positive correlation with CRP ($r = 0.25$, $P < 0.01$) (Table 2). Resistin was not associated with any other study variable, including glucose.

We then performed bivariate regression analyses of adiponectin and of resistin with metabolic factors, inflammatory markers, and vascular reactivity considered as independent variables (Tables 3 and 4). We found a positive correlation of adiponectin with HDL and a negative correlation of adiponectin with triglycerides, CRP, PAI-1, and tPA (Table 3). In addition, there was no association among adiponectin and total cholesterol, LDL, ICAM, ET1, TNF- α , and the vascular reactivity measurements (flow at rest, flow-mediated brachial artery dilation, NID, ACH%, and NNP%). To adjust for potential confounders, we then performed multivariate regression analyses, adjusting for successively introduced covariates: sex, then sex and BMI, and finally sex, BMI, and HbA_{1c}. With adjustment for sex, all of the positive and negative correlations found with the bivariate analyses remained significant. However, after adjusting for both sex and BMI, only the positive association with HDL and the negative association for PAI-1 remained significant (Table 3), indicating that adiponectin may mediate the effect of obesity on triglycerides, CRP, and tPA. Finally, we found a positive correlation of resistin with CRP and a negative correlation with HDL (Table 4), and these associations remained significant after adjusting for sex, BMI, and HbA_{1c}, indicating that the effect of resistin is independent of obesity and glycemic control. We also ran these correlations of adiponectin and resistin with metabolic markers, inflammatory markers, and vascular reactivity, excluding the type 1 diabetic subjects. We found a similar positive correlation of adiponectin with HDL ($r = 0.40$, $P < 0.01$), a negative correlation with PAI-1 ($r = -0.33$, $P < 0.01$), and a negative correlation with BMI ($r = -0.25$, $P < 0.025$). However, the other significant correlations found in the

Table 2—Spearman correlation coefficients among adiponectin, resistin, metabolic and inflammatory markers, and vascular reactivity in subjects at risk of diabetes and in subjects with type 1 and type 2 diabetes

	Adiponectin	Adiponectin:resistin	Resistin	Total cholesterol	HDL	LDL	Triglycerides	ICAM	ET-1	TNF- α	CRP	PAI-1	tPA	BMI	FR	FMD	NID	ACH%
Adiponectin	1																	
Resistin	-0.004	1																
Total cholesterol	0.42*	-0.01	1															
HDL	-0.06	0.11	0.92*	1														
Triglycerides	-0.35*	0.02	0.25*	-0.43*	0.12	1												
ICAM	0.13	0.06	-0.18	-0.22	-0.15	0.07	1											
ET-1	0.17	0.12	-0.09	-0.03	-0.02	0.02	0.37*	1										
TNF- α	-0.07	0.02	-0.03	-0.03	-0.06	0.17	0.001	0.07	1									
CRP	-0.21*	0.25*	0.04	-0.19	0.07	0.16	0.14	0.35*	0.26*	1								
PAI-1	-0.39*	0.08	0.07	-0.41*	0.06	0.52*	0.14	0.15	0.29*	0.28*	1							
tPA	-0.30*	0.07	0.03	-0.40*	0.002	0.51*	0.13	0.21	0.27*	0.36*	0.72*	1						
BMI	-0.32*	0.13	-0.07	-0.40*	-0.01	0.32*	0.24*	0.25	0.26*	0.60*	0.48*	0.48*	1					
FR	-0.12	0.15	-0.11	-0.35*	-0.03	0.16	0.21	0.41*	0.15	0.29*	0.21*	0.27*	0.21*	1				
FMD	-0.01	-0.14	0.01	0.19	-0.05	-0.06	-0.11	-0.16	-0.04	-0.23*	-0.19	-0.34*	-0.13	-0.55*	1			
NID	-0.05	-0.25*	0.07	0.13	-0.04	0.15	-0.24	-0.06	-0.14	-0.27*	0.001	-0.05	0.06	-0.50*	0.57*	1		
ACH%	-0.05	-0.03	0.11	0.13	0.11	-0.09	-0.19	0.02	0.04	0.04	-0.02	-0.03	-0.01	-0.13	-0.11	-0.01	1	
NNP%	0.10	0.06	-0.04	0.11	-0.04	-0.06	-0.13	0.10	-0.05	0.04	-0.03	-0.12	0.10	-0.12	-0.05	-0.05	0.52*	1

FMD, flow-mediated dilation percent increase over baseline (endothelium-dependent reactivity of macrocirculation); FR, resting brachial artery diameter (mm). * $P < 0.01$; $\dagger P = 0.026$; $\ddagger P < 0.025$.

entire group did not remain significant (triglycerides [$r = -0.11$] and CRP [$r = -0.17$]), which was probably due to the smaller n . None of the previously significant correlations of HDL, CRP, and NID with resistin remained significant when we ran these correlations excluding the type 1 diabetic subjects from the entire group.

Finally, we ran all correlations in the entire study sample after statistically controlling for type 1 versus type 2 diabetes status. The previously nonsignificant associations remained unchanged. The significant associations were as follows after controlling for diabetes status: 1) adiponectin versus HDL ($r = 0.27$, $P < 0.01$), versus triglycerides ($r = -0.07$, $P = 0.45$), versus tPA ($r = -0.09$, $P = 0.32$), versus BMI ($r = -0.18$, $P = 0.04$), and versus PAI-1 ($r = -0.022$, $P < 0.026$) and 2) resistin versus HDL ($r = -0.27$, $P < 0.01$), versus CRP ($r = 0.23$, $P < 0.026$), and versus NID ($r = -0.20$, $P = 0.05$), which remained essentially unchanged.

Interventional study

Both atorvastatin and placebo treatment resulted in a decrease in total cholesterol and LDL levels, but the decrease in the atorvastatin group was much greater (Table 5). CRP levels also decreased significantly in the atorvastatin group but did not change significantly in the placebo group. Serum adiponectin levels did not change significantly in either the placebo or active treatment group, and serum resistin levels decreased significantly but to the same extent in both groups (Table 5), indicating that atorvastatin has no specific effect on these two hormones. Similar results were obtained when diabetic and nondiabetic subjects were considered separately.

CONCLUSIONS—Accumulating evidence from animal and human studies shows that adiponectin plays an important role in insulin sensitivity (24–28), inflammation (29), atherogenesis (30,31), lipid metabolism (6,24), and thus influences hyperlipidemia and CAD (29). The association between adiponectin and insulin sensitivity has been established in animal models in which adiponectin administration reversed insulin resistance in lipotrophic mice (25). Adiponectin levels are significantly lower in humans with type 2 diabetes or insulin

Table 3—Bivariate and multivariate regression analyses of serum adiponectin levels on metabolic factors, inflammatory markers, and vascular reactivity*

Parameters	$\beta 1$	$\beta 2$	$\beta 3$	$\beta 4$
Metabolic factors				
Total cholesterol (mg/dl)	0.03	0.02	-0.05	-0.05
LDL cholesterol (mg/dl)	-0.04	-0.04	-0.09	-0.08
HDL cholesterol (mg/dl)	0.37†	0.40†	0.31†	0.31†
Triglycerides (mg/dl)	-0.22†	-0.22†	-0.17	-0.17
Inflammatory markers				
ICAM (ng/ml)	0.17	0.19	0.21	0.19
ET-1 (pg/ml)	0.15	0.15	0.24	0.21
TNF- α (pg/ml)	-0.13	-0.14	-0.06	-0.05
CRP (mg/dl)*	-0.22†	-0.25†	-0.07	-0.20
PAI-1 (ng/ml)	-0.36†	-0.36†	-0.24†	-0.21
tPA (ng/ml)	-0.26†	-0.26†	-0.15	-0.15
Vascular reactivity				
FR	-0.11	-0.14	-0.002	-0.02
FMD	0.004	-0.003	-0.02	0.004
NID	-0.03	-0.05	-0.04	0.01
ACH%	-0.09	-0.12	-0.12	-0.10
NNP%	0.01	0.003	0.04	0.05

$\beta 1$, bivariate standardized linear regression coefficient; $\beta 2$, multivariate standardized linear regression coefficient adjusted for sex; $\beta 3$, multivariate standardized linear regression coefficient adjusted for sex and BMI; $\beta 4$, multivariate standardized linear regression coefficient adjusted for sex, BMI, and HbA_{1c}. FMD, flow-mediated dilation percentage increase over baseline (endothelium-dependent reactivity of macrocirculation); FR, resting brachial artery diameter (in millimeters). *Logarithmic transformation performed before analysis. †P < 0.01; ‡P < 0.025.

resistance (32) and increase in subjects treated with thiazolidinediones (33,34), suggesting that adiponectin plays a role in the thiazolidinedione effect of improving insulin sensitivity and decreasing inflammation. In addition, adiponectin may play a critical role in suppressing the inflammatory response that is associated with atherogenesis, endothelial dysfunction, and ultimately vascular disease (29). In vitro mechanistic studies in human aortic endothelial cells have shown that human recombinant adiponectin in a dose-dependent manner suppresses endothelial expression of adhesion molecules, proliferation of vascular smooth muscle cells, and the transformation of macrophages to foam cells (30). Adiponectin also significantly inhibits macrophage phagocytic activity and suppresses lipopolysaccharide-induced production of TNF- α (29). Animal studies in apolipoprotein E-deficient mice demonstrated that adiponectin suppressed the expression of adhesion molecules, scavenger receptors, and TNF- α levels, which cumulatively resulted in reduced in vivo atherosclerosis (35). Finally, it has recently been reported that circulating adiponectin correlates in-

versely with serum levels of certain inflammatory markers, such as CRP (13). We searched for novel associations between circulating adiponectin and tPA, in addition to confirming known associations with HDL, triglycerides, and PAI-1. Adiponectin's association with HDL and PAI-1 was independent of BMI, which suggests adiponectin may mediate some effects of adiposity, but whether central obesity or other unrecognized pathways might play a regulatory role remains to be elucidated by future studies.

We found that in subjects with diabetes or at risk of developing diabetes, adiponectin is negatively correlated with BMI, triglycerides, CRP, PAI-1, and tPA, suggesting that adiponectin may act as an anti-inflammatory mediator with respect to CRP, PAI-1, and tPA. These findings are consistent with prior data that adiponectin levels correlate negatively with inflammation and endothelial dysfunction (12). In addition to adiponectin's negative association with CRP (13), a molecule known to promote atherogenesis via monocytes and endothelial cells (36), we provide the first description of a negative association between adiponectin and tPA, a molecule known to play a role

Table 4—Bivariate and multivariate regression analyses of serum resistin levels on metabolic factors, inflammatory markers, and vascular reactivity*

Parameters	$\beta 1$	$\beta 2$	$\beta 3$	$\beta 4$
Metabolic factors				
Total cholesterol (mg/dl)	-0.004	-0.01	0.02	0.02
LDL cholesterol (mg/dl)	0.11	0.11	0.13	0.13
HDL cholesterol (mg/dl)	-0.28†	-0.33†	-0.33†	-0.33†
Triglycerides (mg/dl)	0.03	0.04	0.01	0.01
Inflammatory markers				
ICAM (ng/ml)	-0.02	-0.01	-0.06	-0.05
ET-1 (pg/ml)	0.22	0.22	0.23	0.25
TNF- α (pg/ml)	0.01	0.01	-0.02	-0.01
CRP (mg/dl)*	0.28†	0.29†	0.38†	0.39†
PAI-1 (ng/ml)	0.12	0.13	0.10	0.13
tPA (ng/ml)	0.08	0.08	0.03	0.03
Vascular reactivity				
FR	0.17	0.22	0.18	0.17
FMD	-0.13	-0.13	-0.12	-0.12
NID	-0.17	-0.17	-0.18	-0.17
ACH%	0.06	0.07	0.07	0.08
NNP%	0.07	0.06	0.05	0.05

$\beta 1$, bivariate standardized linear regression coefficient; $\beta 2$, multivariate standardized linear regression coefficient adjusted for sex; $\beta 3$, multivariate standardized linear regression coefficient adjusted for sex and BMI; $\beta 4$, multivariate standardized linear regression coefficient adjusted for sex, BMI, and HbA_{1c}. FMD, flow-mediated dilation percentage increase over baseline (endothelium-dependent reactivity of macrocirculation); FR, resting brachial artery diameter (in millimeters). *Logarithmic transformation performed before analysis; †P < 0.01; ‡P < 0.025.

Table 5—Results of changes in response to atorvastatin or placebo

	Atorvastatin		Placebo†	
	Before treatment	After treatment	Before treatment	After treatment
Adiponectin ($\mu\text{g/ml}$)	21.7 \pm 2.3	21.4 \pm 2.2	25.2 \pm 3.5	28.2 \pm 4.1
Resistin (ng/ml)	8.1 \pm 0.8	6.8 \pm 0.6*	8.1 \pm 0.8	6.7 \pm 0.6†
BMI (kg/m^2)	29.5 \pm 1.3	29.3 \pm 1.2	28.8 \pm 1.1	28.9 \pm 1.0
Total cholesterol (mg/dl)	199.8 \pm 7.0	150.7 \pm 5.8†	211.9 \pm 7.0	199.5 \pm 5.3†
HDL (mg/dl)	58.8 \pm 2.3	56.9 \pm 2.3	61.9 \pm 3.3	59.3 \pm 2.9
LDL (mg/dl)	120.4 \pm 5.9	76.7 \pm 4.9†	127.0 \pm 5.6	118.4 \pm 4.6†
Triglycerides (mg/dl)	114.3 \pm 15.0	99.3 \pm 14.5	110.2 \pm 13.0	116.2 \pm 12.9
ICAM (ng/ml)	240.3 \pm 9.2	244.8 \pm 8.6	219.3 \pm 16.9	236.6 \pm 13.3
ET1 (pg/ml)	0.76 \pm 0.05	0.70 \pm 0.04	0.66 \pm 0.08	0.68 \pm 0.07
TNF- α (pg/ml)	3.5 \pm 0.3	3.1 \pm 0.3	2.7 \pm 0.4	3.3 \pm 0.4
CRP (mg/dl)	0.32 \pm 0.05	0.25 \pm 0.04*	0.30 \pm 0.05	0.26 \pm 0.04
PAI-1 (ng/ml)	28.8 \pm 4.0	22.6 \pm 3.3	21.1 \pm 3.0	24.9 \pm 4.3
tPA (ng/ml)	7.7 \pm 0.7	7.2 \pm 0.7	6.6 \pm 0.6	6.4 \pm 0.6

Data are mean \pm SE. Atorvastatin, $n = 34$; placebo, $n = 33$. * $P < 0.025$; † $P < 0.01$; ‡ $P = 0.03$.

in impaired fibrinolysis in humans (37). The negative correlation of adiponectin to PAI-1, which is also associated with insulin resistance and the metabolic syndrome (38,39), provides another possible mechanism for adiponectin in affecting the metabolic syndrome-induced morbidity and mortality. Further analysis of adiponectin adjusting for type of diabetes showed slightly different associations with tPA. These findings need to be confirmed in larger studies.

Resistin, which has recently been proposed to play a role in obesity-mediated insulin resistance (7), has a structure similar to that of proteins that are involved in inflammatory processes (12). More specifically, resistin is identical to FIZZ3 (found in inflammatory zone 3), and resistin-like molecules, such as RELM- α and RELM- β , are identical with FIZZ1 and FIZZ2, respectively. Patterns of expression of FIZZ proteins are very similar to those reported for resistin and RELMs (8,12). In addition, the pattern of expression and physiological functions proposed for these proteins resemble those of other well-known proinflammatory cytokines, such as interleukin-6 and TNF- α , both of which are involved in cardiovascular obesity-related outcomes (40). These findings suggest that resistin/FIZZ3 and RELMs/FIZZ1 and FIZZ2 may be involved in the inflammatory processes associated with obesity (41), but the role of resistin in vascular reactivity or potential associations with inflammatory markers have not been previously studied. In ad-

dition to the known negative correlation between serum resistin and HDL, we report a positive correlation of serum resistin to CRP, and these correlations remained significant after adjusting for type of diabetes, which suggests that the type of diabetes does not influence the associations between resistin levels and these variables. The positive association to CRP also remained significant despite adjustments for sex and BMI, suggesting that resistin's proinflammatory properties may be independent of overall obesity. Larger studies are needed to confirm and extend these finding in healthy and diabetic subjects.

We did not find any correlations between vascular reactivity and adiponectin or resistin in this study. There are two published studies reporting conflicting results regarding adiponectin and vascular function (42,43). More specifically, a recent report showing that hypoadiponectinemia is associated with impaired endothelium-dependent vasodilation (42) conflicts with our findings of no association between adiponectin and any vascular reactivity. Furthermore, the second recently published report stated that adiponectin is associated with endothelium-independent vasodilation, but not endothelium-dependent vasodilation (43). Based on our findings and the findings from these two other groups, we do not feel there is a well-established and reproducible association between vascular reactivity and either adiponectin or resistin levels.

Another topic we addressed in this study was whether statin treatment alters circulating adiponectin and resistin levels. The current understanding of statin efficacy in decreasing the relative risk of major coronary events by 30% has been attributed to several mechanisms, including beneficial effects on plasma lipoprotein levels, endothelial function, plaque architecture and stability, thrombosis, and inflammation, which are seen in addition to and independent of decreased LDL (44–48). Whereas it is enticing to explain some of the vascular and inflammatory effects mediated by atorvastatin possibly via alterations in adiponectin or resistin levels, data from the interventional part of the study show that atorvastatin does not significantly alter serum adiponectin or resistin levels when compared with placebo. These data indicate that other pathways may mediate atorvastatin's effects on insulin resistance and inflammatory processes.

In summary, the findings of this study indicate that adiponectin may have a wide-ranging role in metabolism and inflammation by being associated not only with lipoprotein levels but also inflammatory markers and that some of these associations reflect an underlying association with obesity, whereas others are independent of obesity. However, resistin's associations with inflammatory markers appear to be independent of BMI, suggesting that resistin may have a direct proinflammatory role or mediate its effects via yet to be discovered obesity-

independent mechanisms. Finally, neither vascular reactivity nor lipid profile changes and inflammatory changes produced by therapy with atorvastatin can be attributed to alterations of either adiponectin or resistin levels. Further studies to confirm and extend these observations as well as to elucidate the underlying mechanisms are clearly needed.

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EXHIBIT D

.

Additive Beneficial Effects of Fenofibrate Combined With Atorvastatin in the Treatment of Combined Hyperlipidemia

Kwang Kon Koh, MD, FACC,* Michael J. Quon, MD, PhD,† Seung Hwan Han, MD,* Wook-jin Chung, MD,* Jeong Yeal Ahn, MD,† Yiel-Hea Seo, MD,† In Suck Choi, MD,* Eak Kyun Shin, MD*

Incheon, Korea; and Bethesda, Maryland

OBJECTIVES We compared vascular and metabolic responses (and adverse responses) to statin and fibrate therapies alone or in combination in patients with combined hyperlipidemia.

BACKGROUND The mechanisms of action for statins and fibrates are distinct.

METHODS Fifty-six patients were given atorvastatin 10 mg and placebo, atorvastatin 10 mg and fenofibrate 200 mg, or fenofibrate 200 mg and placebo daily during each two-month treatment period of a randomized, double-blind, placebo-controlled crossover trial with two washout periods of two months' each.

RESULTS Lipoproteins were changed to a greater extent with combined therapy when compared with atorvastatin or fenofibrate alone. Flow-mediated dilator response to hyperemia and plasma high-sensitivity C-reactive protein and fibrinogen levels were changed to a greater extent with combined therapy when compared with atorvastatin or fenofibrate alone ($p < 0.001$, $p = 0.182$, and $p = 0.015$ by analysis of variance [ANOVA], respectively). The effects of combined therapy or fenofibrate alone on plasma adiponectin levels and insulin sensitivity (determined by the Quantitative Insulin-Sensitivity Check Index [QUICKI]) were significantly greater than those of atorvastatin alone ($p = 0.022$ for adiponectin and $p = 0.049$ for QUICKI by ANOVA). No patients were withdrawn from the study as the result of serious adverse effects.

CONCLUSIONS Combination therapy is safe and has beneficial additive effects on endothelial function in patients with combined hyperlipidemia. (J Am Coll Cardiol 2005;45:1649–53) © 2005 by the American College of Cardiology Foundation

High serum cholesterol and elevated low-density lipoprotein (LDL) cholesterol are important risk factors for coronary heart disease. Many patients on statin therapy have initial or recurrent coronary heart disease events despite reductions in LDL cholesterol (1). Interestingly, fibrate therapy, which significantly decreases triglycerides and increases high-density lipoprotein (HDL) cholesterol without reducing LDL cholesterol, is associated with significant decreases in coronary events (2). Moreover, combined therapy with statins and fibrates is more effective in controlling atherogenic dyslipidemia in patients with combined hyperlipidemia than the administration of either drug alone (3). Of concern is the fact that the combination of statins and fibrates is more likely to be accompanied by severe myopathy (4). This limitation is not observed with fenofibrate, and no significant side effects have been reported with combined statin and fenofibrate treatment (3–5).

Coronary heart disease frequently is associated with insulin resistance and metabolic disorders, such as obesity and combined hyperlipidemia. Endothelial dysfunction associated with cardiovascular diseases may contribute to

insulin resistance (6). The effects of statins on insulin resistance are controversial (7,8). Peroxisome proliferator-activated receptor- α activators improve insulin sensitivity in rodents (9). The impact of atorvastatin and fenofibrate therapies on endothelial homeostasis and insulin resistance may differ because the mechanisms underlying the biological actions of these drugs are distinct. Therefore, we investigated whether combined therapy has additive beneficial effects greater than atorvastatin or fenofibrate alone in patients with combined hyperlipidemia.

METHODS

Study population and design. Fifty-six patients with combined hyperlipidemia (total cholesterol ≥ 200 mg/dl and triglycerides ranging from 200 mg/dl to 800 mg/dl) participated in this study. We excluded patients with overt liver disease, chronic renal failure, hypothyroidism, myopathy, uncontrolled diabetes, severe hypertension, stroke, acute coronary events, coronary revascularization within the preceding three months, or evidence of alcohol abuse. Clinical characteristics of the study patients are summarized in Table 1. We administered atorvastatin 10 mg and placebo, atorvastatin 10 mg and fenofibrate 200 mg, or fenofibrate 200 mg and placebo daily during two months in a randomized, double-blind, placebo-controlled crossover trial with three treatment arms (each two months in duration) and two

From the Departments of *Cardiology and †Laboratory Medicine, Gachon Medical School, Incheon, Korea; and the ‡Diabetes Unit, National Center for Complementary and Alternative Medicine, National Institutes of Health, Bethesda, Maryland. This study was partly supported by grant 2002-5 from the Korean Society of Circulation.

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Abbreviations and Acronyms

ANOVA	= analysis of variance
FMD	= flow-mediated dilation
HDL	= high-density lipoprotein
LDL	= low-density lipoprotein
QUICKI	= Quantitative Insulin-Sensitivity Check Index

washout periods (each two months in duration). Patients were observed at 14-day intervals (or more frequently) during the study. To avoid side effects, we measured serum aspartate aminotransferase, alanine aminotransferase, creatine kinase, blood urea nitrogen, and creatinine before and after therapy. Calcium channel or beta adrenergic blockers were withheld for ≥ 48 h before the study. The study was approved by the Gil Hospital Institute Review Board, and all participants gave written, informed consent.

Laboratory assays. Blood samples were obtained at 8:00 AM after an overnight fast before and after each two-month treatment period. Samples were immediately coded so that investigators performing laboratory assays were blinded to subject identity or study sequence. Assays for lipids, glucose, and plasma adiponectin were performed in duplicate by enzyme-linked immunosorbent assay (R & D Systems, Inc., Minneapolis, Minnesota), assays for high-sensitivity C-reactive protein levels by latex agglutination (CRP-Latex(II), Denka-Seiken, Japan), and assays for plasma insulin levels by immunoradiometric assay (INSULIN-RIABEAD II, Abbott Japan, Japan) as described previously (10,11). The Quantitative Insulin-Sensitivity Check Index (QUICKI), a surrogate index of insulin sensitivity, was calculated as follows: QUICKI = $1/[\log(\text{insulin}) + \log(\text{glucose})]$ (12).

Vascular studies. Imaging studies of the right brachial artery were performed using an ATL HDI 3000 ultrasound machine (Bothell, Washington) equipped with a 10-MHz linear-array transducer, on the basis of a published technique (10,11).

Statistical analysis. Data are expressed as mean \pm SEM or median (range, 25% to 75%). After testing data for normality, we used the Student paired *t* or Wilcoxon signed rank test to compare values before and after each treatment (Tables 2 and 3). The effects of the three therapies were

Table 1. Baseline Characteristics of the Study Population

Variables	n = 56
Age	56 \pm 1
Gender, M:F	23:33
Body mass index, kg/m ²	25.5 \pm 0.3
Risk factors	
Current smoking	12 (21)
Ischemic heart disease	12 (21)
Hypertension	38 (68)
Diabetes	9 (16)
Medications	
Beta-adrenergic blockers	23 (41)
Calcium channel blockers	21 (38)

Values are expressed as means \pm SEM or n (%).

Table 2. Effects of Atorvastatin, Combined Therapy, and Fenofibrate on Lipid Levels and Endothelial Function in Patients With Combined Hyperlipidemia

Variables	Atorvastatin (A)			Atorvastatin + Fenofibrate (C)			Fenofibrate (F)			A/C	A/F	C/F
	Baseline 1	Treatment		Baseline 2	Treatment		Baseline 3	Treatment				
Body mass index	25.46 \pm 0.34	25.47 \pm 0.34		25.45 \pm 0.34	25.46 \pm 0.33		25.47 \pm 0.34	25.46 \pm 0.35				
Lipids (mg/dl)												
Total cholesterol	243 \pm 7	173 \pm 5†		240 \pm 6	171 \pm 4†		234 \pm 6	203 \pm 5†		NS	<0.05	<0.05
Triglycerides	301 \pm 23	226 \pm 17†		322 \pm 19	138 \pm 12†		337 \pm 24	150 \pm 10†		<0.001	<0.05	NS
LDL cholesterol	134 \pm 7	81 \pm 5†		128 \pm 6	90 \pm 4†		130 \pm 7	122 \pm 4		<0.001	<0.05	<0.05
Apo B	132 \pm 4	92 \pm 3†		127 \pm 4	89 \pm 3†		128 \pm 4	101 \pm 3†		0.004	<0.05	<0.05
HDL cholesterol	46 \pm 1	46 \pm 2		46 \pm 1	53 \pm 2†		44 \pm 1	54 \pm 2†		<0.001	<0.05	NS
Apo A-I	160 \pm 3	163 \pm 3		162 \pm 3	178 \pm 4†		154 \pm 3	169 \pm 4†		0.005	<0.05	NS
Vasomotor (%)												
FMD (%)	4.70 \pm 0.18	6.38 \pm 0.21†		4.56 \pm 0.19	7.44 \pm 0.22†		4.73 \pm 0.19	6.51 \pm 0.19†		<0.001	NS	<0.05
NTG dilation (%)	13.76 \pm 0.50	14.36 \pm 0.57		14.16 \pm 0.55	14.69 \pm 0.53		12.93 \pm 0.46	13.77 \pm 0.51		0.762		
CRP (mg/l)	1.20 (0.65-2.20)	0.75 (0.40-1.45)*		1.20 (0.70-2.35)	0.60 (0.40-1.10)†		0.80 (0.53-2.03)	0.70 (0.40-1.20)*		0.182		
Fibrinogen (mg/dl)	287 \pm 9	264 \pm 11		297 \pm 7	235 \pm 8†		282 \pm 9	233 \pm 9†		<0.05	NS	NS

Data are expressed as means \pm SEM or median (25th percentile to 75th percentile). There were no significant differences among baseline values. **p* < 0.05, †*p* < 0.001 for comparison with each baseline value. A/C = atorvastatin vs. combined therapy; A/F = atorvastatin vs. fenofibrate; ANOVA = analysis of variance; C/F = combined therapy vs. fenofibrate; CRP = C-reactive protein; FMD = flow-mediated dilation; HDL = high-density lipoprotein; LDL = low-density lipoprotein; NS = not significant; NTG = nitroglycerin.

Table 3. Effects of Atorvastatin, Combined Therapy, and Fenofibrate on Adiponectin Levels and Insulin Resistance in Patients With Combined Hypertension

Variables	Atorvastatin (A)		Atorvastatin + Fenofibrate (C)		Fenofibrate (F)		ANOVA	A/C	A/F	C/F
	Baseline 1	Treatment	Baseline 2	Treatment	Baseline 3	Treatment				
ADP (μg/ml)	3.5 (2.6-5.0)	3.4 (2.6-4.7)	3.4 (2.3-4.7)	3.5 (2.7-5.1)†	3.2 (2.5-5.1)	3.6 (2.6-5.3)†	0.022	<0.05	<0.05	NS
Insulin (μU/ml)	4.34 ± 0.45	4.57 ± 0.58	4.66 ± 0.42	3.54 ± 0.36‡	4.31 ± 0.45	3.40 ± 0.41*	0.012	<0.05	NS	NS
Glucose (mg/dl)	92 ± 3	95 ± 5	91 ± 4	92 ± 3	89 ± 3	89 ± 3	0.896	<0.05	NS	NS
QUICKI	0.411 ± 0.008	0.412 ± 0.008	0.403 ± 0.007	0.430 ± 0.010†	0.419 ± 0.010	0.437 ± 0.009*	0.049	<0.05	NS	NS

Data are expressed as mean ± SEM or median (25th percentile to 75th percentile). There were no significant differences among baseline values. * $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$ for comparison with each baseline value. ADP = adiponectin; QUICKI = Quantitative Insulin-Sensitivity Check Index = $1/\log(\text{insulin}) + \log(\text{glucose})$; other abbreviations as in Table 2.

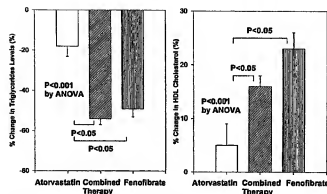


Figure 1. Fenofibrate alone or combined therapy significantly lowered triglycerides and increased high-density lipoprotein cholesterol levels when compared with atorvastatin alone. ANOVA = analysis of variance.

analyzed by one-way repeated measures analysis of variance (ANOVA) or Friedman's repeated ANOVA on ranks by comparing the relative changes in values in response to treatment. Post hoc comparisons between treatment pairs were made with the Student-Newman-Keuls multiple comparison procedure. Pearson or Spearman correlation coefficient analysis was used to assess associations between measured parameters. Comparisons between endothelium-dependent dilation among the three treatment schemes were prospectively designated as the primary study end point. All other comparisons were considered secondary. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

No significant differences among baseline values before each treatment period or carryover effects were noted (Tables 2 and 3).

Effects on lipids. Fenofibrate alone or combined therapy significantly lowered triglycerides and increased HDL cholesterol and apolipoprotein A-I levels when compared with atorvastatin alone (Fig. 1, Table 2).

Effects on vasomotor function. Atorvastatin, combined therapy, or fenofibrate significantly improved the percent flow-mediated dilator response to hyperemia relative to baseline measurements by $46 \pm 9\%$, $81 \pm 12\%$, and $45 \pm 5\%$, respectively (all $p < 0.001$). Of note, combined therapy significantly improved this response more than atorvastatin or fenofibrate alone ($p < 0.001$ by ANOVA) (Fig. 2, Table 2). Brachial artery dilator response to nitroglycerin was not significantly changed from respective baseline values. After combined therapy, improvement in flow-mediated dilation (FMD) correlated with changes in total cholesterol ($r = -0.373$ and $p = 0.005$), triglycerides ($r = -0.288$ and $p = 0.032$), and apolipoprotein B levels ($r = -0.341$, $p = 0.010$).

Effects on acute-phase reactants. Atorvastatin, combined therapy, or fenofibrate significantly lowered plasma high-sensitivity C-reactive protein levels relative to baseline measurements from 1.20 to 0.75 ($p = 0.006$), 1.20 to 0.60

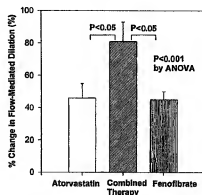


Figure 2. Percent change in flow-mediated dilation from respective pretreatment values after treatment with atorvastatin alone, combined therapy, and fenofibrate alone. ANOVA = analysis of variance.

($p < 0.001$), and 0.80 ± 0.70 mg/l ($p = 0.002$), respectively. However, the magnitude of reduction among the three therapies was similar ($p = 0.182$ by ANOVA). Fenofibrate alone or combined therapy significantly lowered plasma fibrinogen levels relative to baseline measurements (both $p < 0.001$). Of note, combined therapy significantly reduced this more than atorvastatin alone ($p = 0.015$ by ANOVA) (Fig. 3, Table 2).

Effects on adiponectin and insulin resistance. There were significant inverse correlations between baseline adiponectin and baseline triglycerides ($r = -0.277$, $p = 0.039$ before atorvastatin; $r = -0.335$, $p = 0.012$ before combined therapy; and $r = -0.288$, $p = 0.032$ before fenofibrate). There were significant correlations between baseline adiponectin and baseline HDL cholesterol ($r = 0.284$, $p = 0.034$ before atorvastatin; $r = 0.258$, $p = 0.049$ before combined therapy; and $r = 0.353$, $p = 0.008$ before fenofibrate). However, there were no significant correlations between baseline adiponectin and baseline insulin or QUICKI.

Combined therapy or fenofibrate alone significantly increased plasma adiponectin levels relative to baseline measurements from 3.4 to 3.5 ($p = 0.001$) and 3.2 to 3.6 ($p = 0.004$), respectively. These increases were significantly

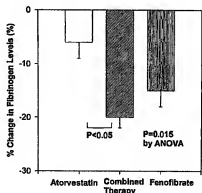


Figure 3. Fenofibrate alone or combined therapy significantly lowered plasma fibrinogen levels relative to baseline measurements. Combined therapy significantly reduced levels more than atorvastatin alone. ANOVA = analysis of variance.

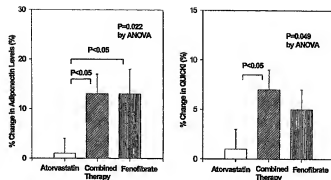


Figure 4. Percent change in adiponectin levels (left) and in Quantitative Insulin-Sensitivity Check Index (QUICKI) (right) from respective pretreatment values after treatment with atorvastatin alone, combined therapy, and fenofibrate alone. ANOVA = analysis of variance.

greater than those observed with atorvastatin alone ($p = 0.022$ by ANOVA) (Fig. 4, Table 3). The three therapies did not have significantly different baseline insulin and glucose levels. However, the magnitude of reduction of insulin with combined therapy was significantly greater than with atorvastatin alone ($p = 0.012$ by ANOVA) (Table 3). Combined therapy or fenofibrate alone significantly increased QUICKI relative to baseline measurements by $7 \pm 2\%$ ($p = 0.003$) and $5 \pm 2\%$ ($p = 0.043$), respectively. These increases with combined therapy were significantly greater than those observed with atorvastatin alone ($p = 0.049$ by ANOVA) (Fig. 4, Table 3). There were significant correlations between percent changes in adiponectin and percent changes in QUICKI ($r = 0.283$, $p = 0.034$) or apolipoprotein A-I ($r = 0.351$, $p = 0.008$), and there were significant inverse correlations between percent changes in adiponectin and percent changes in insulin ($r = -0.332$, $p = 0.013$) after combined therapy. However, there were no significant correlations between percent changes in adiponectin levels and percent changes in triglycerides ($r = 0.085$) or HDL cholesterol levels ($r = -0.048$).

Safety and adverse effects. No patients were withdrawn from the study because of serious adverse effects (Table 4). Elevations in liver and muscle enzymes and gastrointestinal upset were mainly transient and resolved spontaneously after patients finished the study.

Table 4. Adverse Effects of Atorvastatin, Combined Therapy, and Fenofibrate in Patients With Combined Hyperlipidemia

	Atorvastatin (%)	Combined Therapy (%)	Fenofibrate (%)
Liver enzymes 41–120 IU	4 (7)	8 (14)	4 (7)
Liver enzymes 121–136 IU	1 (2)	1 (2)	2 (4)
Creatine kinase 201–629 IU	1 (2)	4 (7)	2 (4)
Gastrointestinal upset	2 (4)	5 (9)	4 (7)

Upper limits of normal of liver enzymes (serum aminotransferases: alanine and aspartate) and creatine kinase are 40 IU and 200 IU, respectively.

DISCUSSION

In patients with combined hyperlipidemia, atorvastatin and fenofibrate therapy alone changed the lipoprotein profiles as expected. We reasoned that distinct biological actions of atorvastatin and fenofibrate therapies on lipoproteins, fibrinogen, adiponectin, and insulin sensitivity may improve endothelium-dependent vascular function by different mechanisms. Indeed, although monotherapy with atorvastatin or fenofibrate significantly improved lipid profiles, endothelial function, inflammatory markers, and insulin sensitivity, combined therapy had additional substantial and significant beneficial effects on these parameters over those seen with monotherapy for either drug. The enhanced vascular reactivity we observed with combination therapy may be the result of both changes in lipoprotein profiles as well as other effects, including pleiotropic actions of statins and actions of fenofibrate to increase nitric oxide production (13). Importantly, no patients were withdrawn from our study as the result of serious adverse effects.

Fenofibrate therapy alone resulted in significant elevation of adiponectin levels, decreased insulin levels, and increased insulin sensitivity (assessed by QUICKI). The present study is the first report demonstrating that fenofibrate therapy can increase adiponectin levels. Adiponectin is an adipose-derived factor that augments and mimics metabolic actions of insulin. Moreover, adiponectin can directly stimulate nitric oxide production from endothelium (14). Therefore, increasing adiponectin levels would be predicted to improve both insulin sensitivity and endothelial function by multiple mechanisms. Interestingly, in contrast to effects of combination therapy on FMD, the beneficial effects of fenofibrate therapy on adiponectin levels, insulin levels, and insulin sensitivity did not increase further with combination therapy. Thus, the benefits with respect to insulin resistance are predominantly the result of fibrate therapy rather than statin therapy, which suggests that improving endothelial function per se (as reflected by FMD) may not completely explain effects of fenofibrate or combined therapy to improve insulin sensitivity. However, combined therapy may reduce insulin resistance by multiple mechanisms such as lipoprotein changes and peroxisome proliferator-activated receptor- α activators. Fenofibrate or combined therapy for two months increased adiponectin levels without a change in body weight or body mass index, which raises the possibility that drug therapy is directly altering adiponectin levels independent of adiposity. It is possible that monotherapy with doses of statins higher than those used in our present study may have additional benefits similar to those we observed with our combined fibrate/statin therapy. However, caution is indicated because recent clinical studies suggest high doses of statins may increase the onset of new diabetes (15). In summary, our study suggests that combined atorvastatin/fenofibrate therapy is safe and has beneficial additive effects, supporting the updated National

Cholesterol Education Program Adult Treatment Panel III guidelines (16).

Reprint requests and correspondence: Dr. Kwang Kon Koh, Director, Vascular Medicine and Atherosclerosis Unit, Gil Heart Center, Gachon Medical School, 1198 Kuwul-dong, Namdong-gu, Incheon, Korea 405-760. E-mail: kwangkong@ghil.com.

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EXHIBIT E

The Effect of 6 Months of Treatment with Pravastatin on Serum Adiponectin Concentrations in Japanese Patients with Coronary Artery Disease and Hypercholesterolemia: A Pilot Study

Kenji Sakamoto, MD; Tomohiro Sakamoto, MD; and Hisao Ogawa, MD, for the Kumamoto Joint Research on Hypercholesterolemia (KOJIROH) Investigators

Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan

ABSTRACT

Background: Pravastatin has been reported to reduce cardiovascular events and mortality in patients with coronary artery disease (CAD). Hypoadiponectinemia is a known risk factor for CAD.

Objective: This study analyzed the effects of short-term pravastatin treatment on serum lipid and adiponectin concentrations in patients with CAD and hypercholesterolemia.

Methods: This was a multicenter, observational pilot study of the effect of 6 months of treatment with pravastatin 10 to 20 mg/d on serum adiponectin concentrations in patients with documented CAD and total cholesterol (TC) levels ≥ 180 mg/dL. Patients from 13 medical centers in Japan were monitored at visits every 4 weeks for assessment of compliance and adverse effects. For the assessment of pravastatin's effects, patients were categorized according to baseline serum adiponectin concentrations: quartile 1 (Q1) = <4.83 $\mu\text{g/mL}$; quartile 2 (Q2) = 4.83 to 7.20 $\mu\text{g/mL}$; quartile 3 (Q3) = 7.21 to 10.38 $\mu\text{g/mL}$; and quartile 4 (Q4) = >10.38 $\mu\text{g/mL}$. The primary end point of the study was the percent change from baseline in adiponectin concentrations at 6 months. Secondary end points were changes in lipids, high-sensitivity C-reactive protein (hsCRP), and glycosylated hemoglobin (HbA_{1c}).

Results: One hundred thirty consecutive patients were enrolled; 11 were excluded and 4 discontinued due to adverse events. Thus, 115 patients were included in the study analyses (83 men, 32 women; mean age, 68 years). No patient had a cardiac event during the 6-month follow-up period. After 6 months of pravastatin treatment, 74 (64.3%) patients had increases in serum adiponectin concentrations. Median (interquartile range) adiponectin concentrations increased significantly from 7.2 (4.8 – 10.4) $\mu\text{g/mL}$ at baseline to 7.8 (5.4 – 11.2) $\mu\text{g/mL}$ after 6 months of pravastatin treatment ($P < 0.001$); the

mean percent increase from baseline was 16.3%. The percent increase from baseline in serum adiponectin concentrations was significantly higher among patients in Q1 (39.3%) compared with those in Q3 (4.5%) and Q4 (6.3%) ($P < 0.003$ and $P < 0.005$, respectively). The relative increase in adiponectin concentrations was significantly correlated with the relative increase in high-density lipoprotein cholesterol (HDL-C) ($r = 0.47$; $P < 0.001$). After 6 months of pravastatin treatment, TC and low-density lipoprotein cholesterol levels had decreased by 14.6% and 23.3%, respectively, and HDL-C levels had increased by 14.0% (all, $P < 0.001$). The change in triglycerides (-13.3%) was not statistically significant. Serum hsCRP levels were significantly decreased from baseline after 6 months of pravastatin treatment ($P < 0.001$). HbA_{1c} did not change significantly.

Conclusion: In this pilot study in Japanese patients with CAD and hypercholesterolemia, 6 months of treatment with pravastatin 10 to 20 mg/d was associated with significant increases in serum adiponectin concentrations. (*Clin Ther.* 2006;28:1012–1021) Copyright © 2006 Excerpta Medica, Inc.

Key words: pravastatin, adiponectin, coronary artery disease, high-density lipoprotein cholesterol.

INTRODUCTION

Adipocytokines are bioactive substances secreted by adipocytes and include growth factors, cytokines, and

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complement factors.^{1,2} Adiponectin is an adipocytokine reported to be the most abundant gene transcript-1 in human adipose tissue. It possesses a signal peptide, collagen-like motif, and globular domain, and has significant homology with collagen X and VIII and complement factor C1q.³ Adiponectin has been reported to have antiatherogenic and antidiabetic functions and is thought to be a beneficial adipocytokine.² Hypoadiponectinemia is seen in patients with coronary artery disease (CAD), and adipocytokine concentrations have been reported to be inversely associated with the severity of CAD.⁴⁻⁷

For nearly a decade, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)-reductase inhibitors (statins) have played an integral role in the pharmacologic management of patients with CAD.⁸ Several studies have found that the beneficial effects of statins are not solely related to their lipid-lowering effects.⁹⁻¹¹ Anti-inflammatory and antioxidative effects are among the so-called pleiotropic effects of statins.^{12,13} There is little published information on the effect of statins on adiponectin concentrations. In one published report,¹⁴ the combination of atorvastatin and rosiglitazone, but not atorvastatin monotherapy, was associated with increases in adiponectin concentrations ($P < 0.05$). Koh et al^{15,16} found that neither atorvastatin nor simvastatin was associated with significant increases in plasma adiponectin concentrations.

As recently reported at the 2006 Scientific Sessions of the American Heart Association,¹⁷ pravastatin prevented new-onset CAD when low-density lipoprotein cholesterol (LDL-C) was only moderately controlled. Pravastatin, but not atorvastatin, has been reported to improve glucose tolerance in patients with type 2 diabetes mellitus^{18,19} and insulin resistance in patients with metabolic syndrome.²⁰

Based on adiponectin's known antidiabetic properties,^{2,21,22} we hypothesized that the effects of pravastatin could be mediated through modification of adiponectin concentrations. The objective of this study was to analyze the effect of short-term pravastatin treatment on serum lipids and adiponectin concentrations in patients with CAD and hypercholesterolemia.

PATIENTS AND METHODS

Study Population

On their initial presentation, patients complained of chest pain typical of myocardial ischemia on effort and/or at rest. For confirmation of qualifying CAD,

all patients underwent diagnostic coronary angiography. CAD was defined as organic stenosis of $>75\%$ and/or a positive provocation test for coronary spasm on intracoronary injection of acetylcholine.²³ Study eligibility also required serum total cholesterol (TC) levels ≥ 180 mg/dL.

The main exclusion criteria were age <20 years, scheduled coronary artery bypass graft surgery, allergy to pravastatin, use of any lipid-lowering drug, and presence of a severe hepatic (eg, cirrhosis) or renal disorder or other severe acute or chronic concomitant disease (eg, severe congestive heart failure [New York Heart Association (NYHA) class III-IV], malignant diseases) that seriously affected the patient's general health.

Study Design and Procedures

The KOJIROH (Kumamoto Joint Research on Hypercholesterolemia) study was a multicenter, uncontrolled, observational pilot study performed in accordance with the ethical principles set forth in the Declaration of Helsinki and approved by local ethics committees or institutional review boards. Written informed consent was obtained from all patients.

Between February 2004 and June 2005, consecutive eligible patients with CAD and hypercholesterolemia were enrolled at 13 medical centers in Japan. All patients received pravastatin 10 to 20 mg/d for 6 months. The dosage was determined by the patients' physician according to baseline TC levels. Patients visited their respective outpatient clinics every 4 weeks during the study and were interviewed concerning compliance and adverse effects.

Peripheral blood samples were obtained after a 12-hour overnight fast at baseline and after 6 months of therapy. Serum was separated by centrifugation at 3000 rpm at 4°C for 10 minutes and stored at -80°C until collected by the central laboratory (SRL Inc., Tokyo, Japan), which performed assays of the lipid profile, glycosylated hemoglobin (HbA_{1c}), and high-sensitivity C-reactive protein (hsCRP) levels. TC, LDL-C, and triglyceride (TG) levels were measured using enzyme assays. High-density lipoprotein cholesterol (HDL-C) levels were measured by cholesterol oxidase assay of the supernatant from the precipitate of non-HDL lipoproteins. HbA_{1c} values were determined using a latex agglutination-turbidimetric immunoassay, and hsCRP levels were quantified using immunonephelometry.²⁴

A portion of the samples was delivered to Kumamoto University for examination of adiponectin concentrations by enzyme-linked immunosorbent assays, as described elsewhere.²⁵ The median normal value for adiponectin was 7.2 µg/mL, with 90% of normal values <13.3 µg/mL and a lower limit of detection of 1.7 µg/mL. The interassay CV ranged from 3.6% to 3.3%, and the intra-assay CV ranged from 5.8% to 4.6% (CV data provided by Otsuka Pharmaceutical Co., Tokyo, Japan).

Study Measures

The primary end point of the study was the percent change from baseline in adiponectin concentrations at 6 months. This was calculated as

$$\frac{[(\text{Month 6 adiponectin} - \text{baseline adiponectin}) / \text{baseline adiponectin}] \times 100}{}$$

For more detailed assessment of the effects of pravastatin, patients were categorized according to baseline serum adiponectin concentrations as follows: quartile 1 (Q1) = <4.83 µg/mL; quartile 2 (Q2) = 4.83 to 7.20 µg/mL; quartile 3 (Q3) = 7.21 to 10.38 µg/mL; and quartile 4 (Q4) = >10.38 µg/mL. Secondary end points were changes in lipids, hsCRP, and HbA_{1c}. In addition, adiponectin concentrations were compared in those taking and not taking an angiotensin-receptor blocker (ARB) and in those with and without a previous myocardial infarction (MI).

Safety was assessed in terms of the incidence of treatment-related and treatment-emergent adverse events and changes in laboratory safety parameters, vital signs, and physical findings. Adverse events were collected by the study physicians, who determined the relationship of the event to pravastatin treatment.

Statistical Analysis

For the primary end point, percent change from baseline in serum adiponectin concentrations at 6 months, we anticipated 40% net increases in adiponectin at 6 months. Assuming a 5% dropout rate, we estimated that a sample size of 100 patients treated with pravastatin would provide ≥80% power to detect a significant change during 6 months of treatment, using a 2-tailed α of 0.05 and an estimated SD of 10%.

All statistical analyses were conducted using StatView for Windows version 5.0 (SAS Institute Inc., Cary, North Carolina). Comparisons of serum param-

eters (TC and LDL-C) before and after pravastatin treatment and comparisons of adiponectin concentrations in those taking and not taking an ARB and those with and without a previous MI were performed using paired or unpaired *t* tests. Because of the skewed distribution of observed values for each parameter, concentrations of HDL-C, TG, hsCRP, and adiponectin were compared using the nonparametric Wilcoxon rank sum test. The normalcy of the distribution of the data was tested using the Kolmogorov-Smirnov test. A *P* value >0.05 indicated that the observed distribution of variables was not statistically different from the normal distribution. Correlations between serum parameters were analyzed using a Pearson product-moment correlation test. Differences with a *P* value <0.05 were considered statistically significant.

Data are presented as mean (SD) or median (interquartile range [IQR]). Differences in the frequencies of the 4 serum adiponectin quartiles were analyzed using the *F* distribution test. Continuous data were compared by 1-way analysis of variance followed by the Scheffé *F* test.

RESULTS

Of the 130 patients initially enrolled, 7 declined to participate and 4 were excluded for violation of the inclusion criteria (2 with severe liver cirrhosis, 1 with gastric cancer, and 1 with severe congestive heart failure [NYHA class III]). Four patients discontinued the study because of adverse events: 1 case of severe toxic eczema, which was considered related to pravastatin, and 1 case each of mild eczema, increased aminotransferases, and general fatigue, which were considered possibly related to pravastatin. Thus, 115 patients were included in the study analyses (Figure 1). Their baseline characteristics are summarized in Table I. Changes from baseline in the study end points are summarized in Table II.

All patients were compliant with study medication. No patient had a cardiac event during the 6-month follow-up period.

Changes in Serum Lipid Levels

TC levels decreased 14.6%, from a mean (SD) of 209.5 (26.3) mg/dL at baseline to 178.8 (26.5) mg/dL after 6 months of treatment (*P* < 0.001). LDL-C levels decreased 23.3%, from 133.9 (22.3) mg/dL at baseline to 102.7 (20.8) mg/dL at 6 months (*P* < 0.001). The -13.3% change in TG levels from a median (IQR)

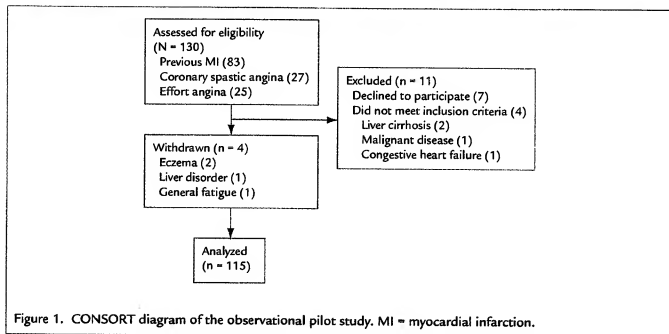


Table I. Baseline characteristics of study patients (N = 115).

Characteristic	Value	Characteristic	Value
Age, mean (SD), y	68 (10.5)	Cardiovascular risk factors, no. (%)	
Sex, no. (%)		Hypertension	76 (66.1)
Male	83 (72.2)	Previous myocardial infarction	63 (54.8)
Female	32 (27.8)	Current smoking	40 (34.8)
Body mass index, mean (SD), kg/m ²	24.1 (3.1)	Coronary spastic angina	27 (23.5)
		Exertional angina	25 (21.7)
		Diabetes	22 (19.1)

Table II. Values at baseline and after 6 months of therapy with pravastatin 10 to 20 mg/d (mean [SD] dosage, 13 [6] mg/d).

Parameter	Baseline	6 Months
Lipids, mg/dL		
TC, mean (SD)	209.5 (26.3)	178.8 (26.5)*
LDL-C, mean (SD)	133.9 (22.3)	102.7 (20.8)*
HDL-C, median (IQR)	43.0 (37.0–49.0)	49.0 (43.0–54.0)*
TG, median (IQR)	128.0 (91.0–168.8)	111.0 (78.0–162.8)
HbA _{1c} , median (IQR), %	5.1 (4.9–5.6)	5.2 (4.8–5.6)
hsCRP, median (IQR), mg/L	1.1 (0.5–3.3)	0.6 (0.3–2.2)*
Adiponectin, median (IQR), µg/mL	7.2 (4.8–10.4)	7.8 (5.4–11.2)*

TC = total cholesterol; LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol; IQR = interquartile range; TG = triglycerides; HbA_{1c} = glycosylated hemoglobin; hsCRP = high-sensitivity C-reactive protein.
*P < 0.001.

of 128.0 (91.0–168.8) mg/dL at baseline to 111.0 (78.0–162.8) mg/dL at 6 months was not statistically significant. HDL-C levels increased 14.0%, from a median of 43.0 (37.0–49.0) mg/dL at baseline to 49.0 (43.0–54.0) mg/dL at 6 months ($P < 0.001$). The percent changes in serum lipids are illustrated in Figure 2.

Changes in Serum Adiponectin Concentrations

Median (IQR) serum adiponectin concentrations were 7.2 (4.8–10.4) $\mu\text{g/mL}$ at baseline. Baseline adi-

ponectin concentrations were significantly correlated with age ($r = 0.399$; $P < 0.001$), body mass index ($r = -0.313$; $P < 0.001$), and TG levels ($r = -0.301$; $P = 0.001$). Adiponectin concentrations were numerically but not statistically significantly increased in 74 (64.4%) patients, with a normal distribution (Figure 3). After 6 months of pravastatin treatment, serum adiponectin concentrations had increased by 16.3% to 7.8 (5.4–11.2) $\mu\text{g/mL}$ ($P < 0.001$) (Figure 4).

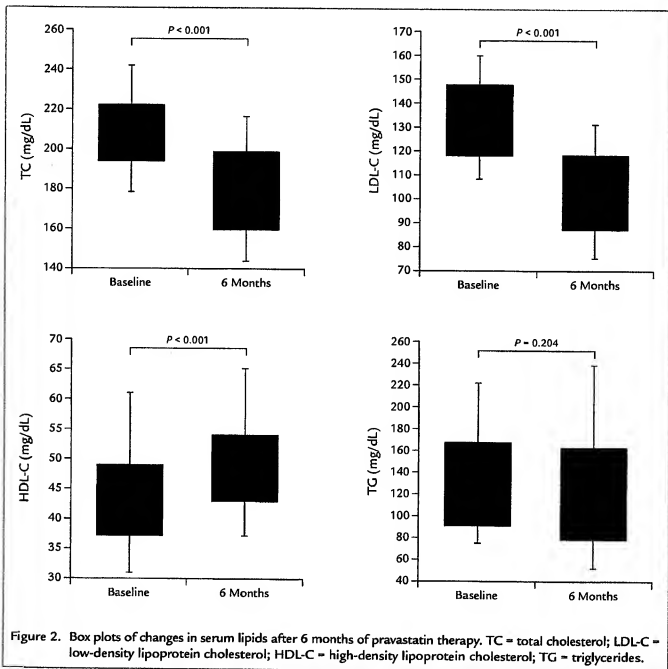


Figure 2. Box plots of changes in serum lipids after 6 months of pravastatin therapy. TC = total cholesterol; LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol; TG = triglycerides.

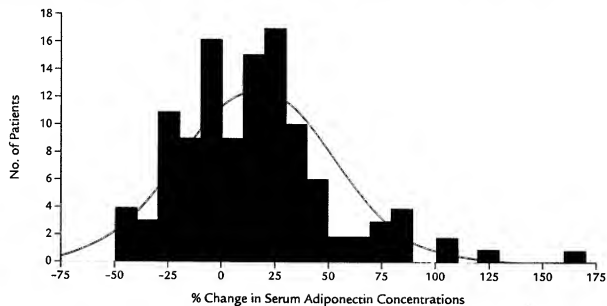


Figure 3. Histogram analysis of percent changes in serum adiponectin concentrations after 6 months of pravastatin therapy.

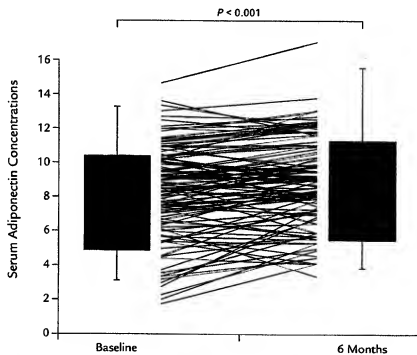


Figure 4. Plot of changes in serum adiponectin concentrations at baseline and after 6 months of pravastatin therapy.

The percent increases in serum adiponectin concentrations in the 4 quartile groups were 39.3% in Q1, 15.9% in Q2, 4.5% in Q3, and 6.3% in Q4. The difference in increase was significant for Q1 compared with Q3 and Q4 ($P < 0.003$ and $P < 0.005$, respectively) (Figure 5). As to the correlation between the relative changes in adiponectin and lipid concentrations, only the correlation with HDL-C was statistically significant ($r = 0.47$; $P < 0.001$) (Figure 6).

Fifty (43.5%) patients were using ARB therapy at baseline, and 65 (56.5%) were not. There was no significant difference in the percent increase in adiponectin concentrations between patients who were taking an ARB (15.5%) and those who were not (16.9%). No patients initiated ARB treatment during the 6-month study period. Sixty-three (54.8%) patients had a previous MI (including 12 with acute MI). In the 12 patients with acute MI, the mean interval between acute events and the start of pravastatin therapy was 9.4 days. There was no significant difference in the percent increase in adiponectin concentrations in those with a previous MI (19.4%) and those without a previous MI (12.5%).

Changes in Serum hsCRP Levels and HbA_{1c} Values

Serum hsCRP decreased significantly from a median (IQR) of 1.1 (0.5–3.3) mg/L at baseline to 0.6 (0.3–2.2) mg/L after 6 months of pravastatin treatment ($P < 0.001$) (Figure 7). These changes were not significantly correlated with the changes in adiponectin concentrations ($r = 0.149$).

Overall, serum HbA_{1c} values were not significantly changed at the end of 6 months. The baseline median (IQR) value was 5.1% (4.9–5.6), and the value at 6 months was 5.2% (4.8–5.6). However, in patients with baseline HbA_{1c} values $\geq 7.0\%$, the change at 6 months was statistically significant: from 7.8% (7.6–8.6) to 6.7% (5.8–7.5) ($P = 0.036$).

DISCUSSION

In this pilot study, therapeutic doses of pravastatin increased serum adiponectin concentrations after 6 months. Some statins have pleiotropic effects in addition to their effects on serum lipids.^{9–11} These effects include induction of endothelial nitric oxide synthase^{13,26}, inhibition of adhesion molecules and chemotactic factors (eg, vascular cell adhesion molecule-1,²⁷ intercellular adhesion molecule-1,²⁸ monocyte chemoattractant protein-1²⁹), and inhibition of matrix metalloproteinase³⁰ and type 1 plasminogen activator inhibitor.³¹

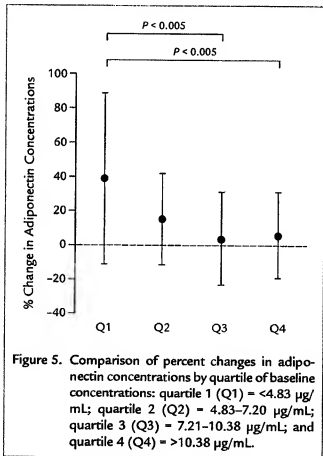


Figure 5. Comparison of percent changes in adiponectin concentrations by quartile of baseline concentrations: quartile 1 (Q1) = <4.83 $\mu\text{g/mL}$; quartile 2 (Q2) = 4.83–7.20 $\mu\text{g/mL}$; quartile 3 (Q3) = 7.21–10.38 $\mu\text{g/mL}$; and quartile 4 (Q4) = >10.38 $\mu\text{g/mL}$.

Serum concentrations of adiponectin were increased by the HMG-CoA-reductase inhibitor pravastatin. In 2 randomized, double-blind, placebo-controlled, crossover trials in patients with combined hyperlipidemia or hypertensive hypercholesterolemia (excluding patients with recent acute coronary syndrome), 2 months of monotherapy with atorvastatin 10 mg¹⁵ and simvastatin 20 mg¹⁶ did not significantly increase plasma adiponectin concentrations. The discrepancy between the findings of these studies and the present trial may be related to drug-specific effects of pravastatin or to differences in study populations and/or designs (including durations). Head-to-head comparative trials in the same populations are necessary to clarify differences in the effects of individual statins on adiponectin concentrations.

The precise mechanism(s) for the increase in adiponectin concentrations associated with pravastatin therapy is unknown. One explanation is that pravastatin may influence the production of adiponectin from adipocytes. We believe that pravastatin does not have a direct effect on adiponectin synthesis and secre-

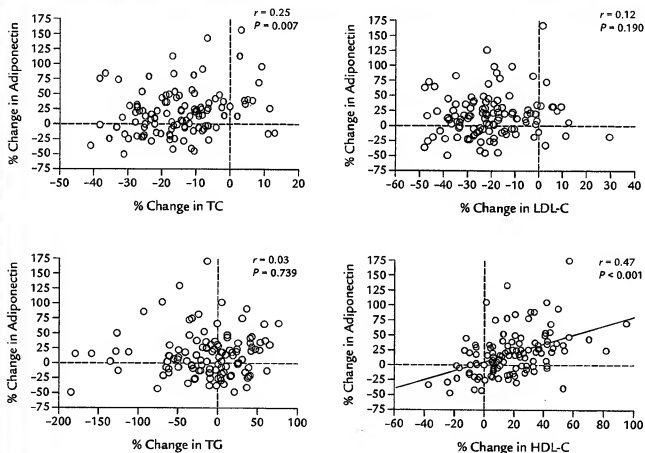


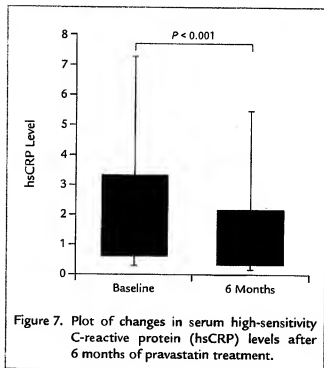
Figure 6. Correlations between percent changes in serum adiponectin concentrations and percent changes in serum lipid levels. TC = total cholesterol; LDL-C = low-density lipoprotein cholesterol; TG = triglycerides; HDL-C = high-density lipoprotein cholesterol.

tion from adipocytes; therefore, the drug may alter adiponectin concentrations indirectly through actions on a remote organ with a high affinity for pravastatin. From this viewpoint, the liver could be the most likely effector organ, and humoral factors produced by the liver might regulate adiponectin production in adipocytes. Pravastatin is the most hydrophilic of the statins and has more effect on hepatocytes than the other lipophilic statins.³²

In the present study, the changes in HDL-C were significantly correlated with changes in serum adiponectin concentrations during pravastatin therapy. In a study in 60 nondiabetic patients with low HDL-C and metabolic syndrome, 30 of whom received pioglitazone 30 mg for 6 weeks and 30 of whom were controls, Szapary et al.³³ reported that pioglitazone treatment was associat-

ed with a 15% increase in HDL-C levels (11% increase in pioglitazone group, 4% decrease in controls; $P < 0.001$). The HDL-C-raising effect was not correlated with changes in insulin resistance but only with changes in adiponectin concentrations ($r = 0.34$; $P = 0.01$); therefore, the authors concluded that pioglitazone may exert its effects on HDL-C indirectly via adiponectin. The strong correlation between changes in HDL-C and adiponectin concentrations in the present study may indicate some mediation of pravastatin's HDL-C-raising effects through changes in adiponectin, as theorized by Szapary et al.

This study had several limitations, including a small sample size, short duration, and lack of a comparator group. Lifestyle changes were not monitored or controlled.



CONCLUSIONS

In this pilot study, pravastatin 10 to 20 mg/d for 6 months was associated with increases in serum adiponectin concentrations in these Japanese patients with CAD and hypercholesterolemia. These preliminary findings require further study in larger, prospective, randomized, double-blind, controlled, comparative trials.

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Address correspondence to: Tomohiro Sakamoto, MD, Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan. E-mail: tom@kumamoto-u.ac.jp